GENETICS OF THE RESISTANCE TO AND PRODUCTION OF H_2O_2 IN *STREPTOCOCCUS PNEUMONIAE*

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CHAPTER I

INTRODUCTION

Hydrogen peroxide (H_2O_2), a reactive oxygen species, is capable of causing damage to cellular components, such as DNA, proteins, and lipids (36, 49, 81). This compound is found in all living cells undergoing aerobic respiration, as redox reactions take place. Normally, aerobic microorganisms are protected from the cytotoxic effect of H_2O_2 by the enzyme catalase which neutralizes it to oxygen and water, but *Streptococcus pneumoniae* does not encode for catalase (81). Involvement of H_2O_2 together with pneumolysin, a cytolysin enzyme, in virulence has been shown to mediate pneumococcal meningitis (13).

Production of H_2O_2 by pyruvate oxidase (SpxB) in *S. pneumoniae* can reach millimolar levels, which is 10^3-fold higher than the concentration required for inhibiting *Escherichia coli* growth and capable of extending toxic effects on many other species of bacteria and even eukaryotic cells (13, 79, 81). In a H_2O_2 sensitivity test, DP1004, an Rx1 strain of *S. pneumoniae*, showed a 10-fold increased resistance to H_2O_2 when pregrown in lactose than when compared to other sugars (Fig. 1). The mechanisms by which *S. pneumoniae* is able to demonstrate resistance to both endogenously generated H_2O_2 and

lactose-induced protection to H_2O_2 are unknown. H_2O_2 resistance has been shown to depend on the *spxB* gene, and its enzymatic activity (81). Other H_2O_2 protective protein homologs, found by amino acid homology studies, such as thioredoxin reductase (*trxB*), glutathione reductase (*gor*), glutathione peroxidase (*basA*), and glyoxalase (spr0643) were identified, but the function of each has not been determined. Nicotinamide adenine dinucleotide oxidase (*nox*) has been shown to confer protection to the bacterium from oxidative stress by reducing O_2 to H_2O (4, 81). Mutation of *spxB* abolishes H_2O_2 resistance, even in a lactose pregrown mutant, demonstrated by the H_2O_2 sensitivity test (118). The Mechanism of catabolite-regulated gene expression in *S. pneumoniae* is shown to have very similar properties to enteric Gram negative bacteria, the *lac* operon (71, 107). Catabolite-activator protein (CAP of *E. coli*) or catabolite control protein (CCP of *S. pneumoniae*) are DNA-binding proteins governing transcription of catabolic operons. The present study was designed to determine several aims:

Aim I: Analysis of possible mechanism of lactose-regulated H_2O_2 resistance

I. Does the CCP protein regulate the expression of both *lac* operon as well as the H_2O_2 resistance genes?

II. What co-factor activates the CCP or a protein with a similar function?

III. Which genes are involved in the H_2O_2 resistance?

Aim II: Genetical analysis of *spxB*

I. Does pneumococcal SpxB-mediated H_2O_2 resistance have a correlation with the H_2O_2 production?

II. Do any of the catabolite-regulated genes affect the H_2O_2 production?

III. Does catabolite regulation play a role in SpxB-mediated H_2O_2 resistance?

IV. What are the factors that regulate SpxB's activity?

Grown in	% survival
Lactose	**17.65 ± 2.47**
Galactose	**1.14 ± 0.59**
Glucose	**1.15 ± 0.74**
Fructose	**1.36 ± 0.74**
Maltose	**1.46 ± 0.59**
Mannose	**1.66 ± 0.74**
Xylose	**1.90 ± 0.93**
Sucrose	**1.96 ± 1.05**

Figure 1. Effect of exogenous H_2O_2 on pneumococcal cells grown in various carbohydrates. Pneumococcal wild-type cells were grown microaerobically in various sugars shown above in $CATP_{Sugar}$ at 37˚C. At OD_{550} = 0.1, the cells were treated with 10 mM exogenous H_2O_2 and incubated at 37˚C for 30 min before plating on CATPGlu agar plates. Survival was given in percentage (% survival rate). An equal volume of sterile water as in 10 mM H_2O_2 was used to treat cells for a control (118).

CHAPTER II

REVIEW OF LITERATURE

S. pneumoniae, discovered more than 100 years ago, is one of the earliest-known human pathogens (45). DNA, as the genetic material of all living things, was first determined in this bacterium by Avery's group (5). This Gram positive, lancet-shaped, aero-tolerant diplococcus, commonly known as pneumococcus, is a member of the lactic acid bacteria (1, 81). The sequenced genome, 2.04 mega bases in length, is composed of low G-C, and has allowed the identification of several virulence factors by homology comparison to other pathogens (46). *S. pneumoniae* is the major cause of otitis media, pneumonia, bacteremia, and meningitis in immuno-compromised hosts (13, 53, 81, 86, 117). *S. pneumoniae* is harbored inside hosts of all ages in the nasopharynx. Around 5 million children worldwide under the age of 5 are killed annually by pneumonia, with *S. pneumoniae* being the leading cause of death (53). Otitis media caused by this bacterium is also common around 5 years of age with approximately 7 million cases annually in the United States. There are about 50,000 cases of bacteremia and 3000 cases of meningitis found annually in the US (53). Together, this pathogen is recognized as the main cause of death in the United States compared with any other bacterial pathogen (53).

Pathogenesis of *S. pneumoniae*

Many proteins or enzymes involved in the pathogenesis of Gram-positive pathogens are found on the cell surface (53). Even though advanced instruments available in genomic study have allowed the identification of many virulence factors, the full picture of the mechanism involved in pneumococcal pathogenesis has still yet to emerge, and allow a greater understanding of the deadliest human pathogen (53).

Some cell surface proteins of *S. pneumoniae*, capable of causing virulence to the pathogen, identified by antibody recognition, are pneumococcal surface protein (*pspA*), hyaluronate lyase (*hyl*), autolysin (*lytA* amidase), pneumolysin (*ply*), and neuraminidase (*nanA* or *nanB*).

Capsule

Protection of *S. pneumoniae* by the capsule, a compound of polysaccharides found outside of the pneumococcal cell wall, from human phagocytosis renders this pathogen clinically important (1, 2, 47). Encapsulated strains are found to require a lower dose (50%) of bacteria for disease development in the host than the non-encapsulated strains; furthermore, capsulated strains are much more virulent than their counterpart (111). Currently, there are more than 90 serotypes of pneumococcal capsule, which have been determined to have no cross immune-reactivity between each other (97). Pneumococcal cells are protected from phagocytosis by the capsule which blocks the host complement system from accessing the bacterial cell wall (1, 2, 47). Capsules of various types have been shown to be associated with different degrees of virulence (57). Virulence of *S. pneumoniae* has also been shown to depend on the presence of other genetic background which together contributes to the full pathogenesis of *S. pneumoniae* (57).

Pneumococcal surface protein A

The PspA enzyme (67 – 99 kDa) is found on the cell wall of the bacterium. It stabilizes the capsule, and has structural and antigenic variability among different pneumococcal strains (52). The compound protects the pathogen from host phagocytosis and C3-mediated of complement clearance (14). The presence of this protein homolog in other microbes, such as *Streptococcus pyogenes*, *Plasmodium falciparum*, *Enterococcus*

faecalis, and *Staphylococcus aureus*, likely offers a similar defense mechanism to these bacteria (53).

Pneumococcal surface antigen A

PsaA enzyme (~37 kDa) is present on the cell wall of the bacterium (A). Attenuation of *pspA* has been shown to confer avirulence to the mutant in both intraperitoneal and intranasal challenges model (11). Sequence analysis suggests that PspA belongs to the family of ABC-transporter, and it is responsible for the uptake of Zn^{2+} and Mn^{2+} into a pneumococcal cell, suggesting possible role of the ions in pneumonoccal virulence. This observation was confirmed by the findings that Mn^{2+} is required by pneumococcal superoxide dismutases (SOD), to detoxify hydrogen peroxide made during aerobic growth by the bacterium, as well as to induce other pneumococcal virulence related genes (55, 88). On the other hand, the role of Zn^{2+} is not clearly known. Low concentration of Zn^{2+} in both the femur and the plasma of mice treated with no Zinc in the diet was shown to lead to a reduced response to the PsaA antigen in the mice, resulting to high risk of pneumococcal infection and death (101). Amino acid sequence analysis has shown homology with putative fimbrial adhesions of *Streptococcus sanguis* and *Streptococcus parasanguis* (11).

Hyaluronate lyase

Hyl enzyme (107 kDa) is a surface protein, and it belongs to a broader group of enzymes known as hyaluronidases. Full virulence of *S. pneumoniae* requires the presence of this enzyme. Activation of Hyl by Ca^{2+} causes degradation of hyaluronan which is part of the extracellular matrix components (60, 84, 85); consequently, this enzyme promotes tissue permeability for pneumococcal invasion (57). In addition this enzyme is also present as a released compound, and is suggested to facilitate endolysis and promote pneumococcal invasion to the host (9, 53). Hyaluronan has been shown to have connections with many biological processes like fertilization, embryonic development, cell migration, differentiation, wound healing, inflammation, growth, metastasis of tumor cells and defense mechanisms (53, 105, 110). Homologs of this protein are found in *S. aureus*, *Propionibacterium acnes*, and *Streptococcus agalactiae*, which are also known human pathogens (53, 82, 83).

Neuraminidase

This enzyme is present in different forms (NanA 107 kDa, NanB 75 kDa), and is located on the surface of pneumococcal cells (15). The different sizes of neuraminidase favor a certain pH for the enzymatic activity; NanA pH 7, NanB pH 5 (10). It cleaves sialic acid from the host cell surface glycans, such as mucin, glycolipids, and glycoproteins (53). The role of neuraminidase is suggested to enhance colonization of this bacterium by exposing the host cell due to the cell structure degradation, but it is yet

to be determined (58, 61, 91). A protein homolog is found in *Salmonella enterica* serovar Typhimurium although it has less than 50% amino acid similarity (53).

Autolysin

Autolysin is common in all bacterial cells. It is responsible for degradation of bacterial peptidoglycan during cell growth, turn over, and cell separation (89, 108). In a middle ear infection test with a chinchilla otitis media model, this enzyme was shown to play a major role in causing otitis media (93). Virulence of this enzyme is connected to its ability to release a cytoplasmic cytolysin, the pneumolysin (54, 69). Under laboratory conditions, pneumococcal autolysin enzyme, N-acetylmuramoyl-L-alanine amidase (~36 kDa), encoded by *lytA*, is found to cause autolysis during stationary phase and competence development. In addition, lysozyme, a host defensive component, is shown to enhance autolysin production and promote release of pneumolysin (1, 69).

Pneumolysin

Pneumococcal Ply (53 kDa) is a cytoplasmic enzyme found in all clinical pneumococcal strains known today (73, 74, 75). It is a choresterol-dependent cytolysin (53). The steps of cytolysis by this enzyme include, binding to the host cell cytoplasmic membrane choresterol, inserting into the membrane, and then, pore formation. As a result, target cells can range from ciliated bronchial epithelial cells to pulmonary endothelial cells. These cells undergo lysis, causing pneumonia and bacteremia (53, 87,

92, 100). In addition, it is also found to be toxic to phagocytes and immune cells, resulting in a compromised immune system and pathogenic infection (53). Protein homologs are found in *Clostridium perfringens*, *Aeromonas hydrophilia*, *Staphylococcus aureus*, and *Bacillus anthracis* (53).

Phase variation

Different ecological characteristics of *S. pneumoniae* are determinded by the niches in the host which are different by oxygen levels. The human nasopharynx is highly aerated, while most of the other sites provide an anaerobic environment. In order to adapt to the different conditions in the host during infection, this bacterium carries out the process of phase variation. The difference in phases has been corresponded to variations in the colony morphology (opaque or transparent), and the transparent variant has been connected with an increased ability to attach to the human nasopharynx, vascular endotheliar cells, and cytokine-stimulated type II lung cells (113). Consequently, the bacterium proliferates and increased bacterial density is found at the site of attachment. On the contrary, the opaque variant is found to be more adaptable and cause systemic infections after intraperitoneal inoculation into mice (114).

Treatment of infections due to pneumococcus

Pneumococcal infection is commonly treated by Beta-lactam antibiotics. Currently, the bacterium is becoming more difficult to treat, mainly due to some

pneumococcal strains that have gradually gained resistance to antibiotics (7, 22, 23, 76). Efforts to explore different ways to treat pneumococcal infections, such as vaccines derived from pneumococcal surface proteins, recognized by the surface enzyme signature sequence LPXTG (15, 34, 35, 94), are on going (8, 10, 12, 53, 65). Even though the number of vaccines has increased by ~50% since 1983, vaccination is ineffective for children younger than 2 years due to a poor immunologic response (53). In addition, conjugate vaccines, developed by chemically linking bacterial polysaccharides to an enzyme, have provided a protection to all recipients, but it still shows limitation because *S. pneumoniae* occasionally goes through modification of the capsule and the high production costs of vaccine (45, 64).

Mechanisms of catabolite regulated gene expression in other bacteria

To date, the role of sugar in pneumococcal pathogenesis is not clearly known. However, the mechanisms of gene regulation governed by sugar have been illustrated in other bacteria.

CAP in *Escherichia coli*, *lac* operon

A well studied mechanism where gene expression is regulated by carbohydrate metabolism is available in *E. coli*. In this bacterium, the gene regulation is governed by the phosphate-transferase system (PTS). This multi-enzyme complex system distributes part of the enzymes on the cell surface of the bacterium. Attachment of a specific sugar to

these surface enzyme receptors triggers cellular activities to take place and regulate transcription of the *lac* operon. In brief, the PTS enzyme IIA^{Glc} activates adenylate cyclase by phosphorylation; consequently, it catalyzes production of the cyclin adenine monophosphate (cAMP). The cAMP is then bound with a catabolite-activator protein (CAP, equivalent to CCP) to form the cAMP-CAP complex. Subsequently, the complex binds to CAP region located upstream of promoter of the *lac* operon. As a result, an interaction between cAMP-CAP complex and RNA polymerase takes place, and this is found to enhance transcription of the operon (59, 78, 107).

CCP in Gram-positive bacteria

However, in the low G-C Gram-positive bacteria, it is slightly different. In the presence of lactose, the PTS serves as the major transporter to bring lactose in, and does not have IIA^{Glc}, and cAMP is found to activate transcription of the *lac* operon. In this system, a non-lactose carbohydrate allows phosphorylation of the Histidine-containing phosphocarrier protein (HPr) at the serine residue, HPr (Ser-P) by HPr kinase/phosphorylase. The HPr (Ser-P) is then bound with a Ccp to form the complex HPr (Ser-P) – Ccp. Subsequently, this complex then binds to the catabolite-responsive element (cre, equivalent to CAP), located at upstream of the promoter region of the *lac* operon and this is found to prevent transcription of the operon (59, 78, 107).

Together, the information suggested that there are catabolic operons that are responsible for H_2O_2 resistance in *S. pneumoniae*, and transcription of the catabolic

operons could be regulated by the same mechanism.

Pyruvate oxidase (*spxB*) as one of the virulence factors in *S. pneumoniae*

This 1.8 kb gene is shown to be present in *S. pneumoniae* in a monocistronic operon by Northern blots (81, 19). A homologous study by amino acid alignment did not show any match to *E. coli* enzymes. It is the major H_2O_2-producing agent in *S. pneumoniae* (81).

H_2O_2 production by SpxB

As a nasopharyngeal inhabitant, *S. pneumoniae* possesses the enzymes lactate oxidase (Lox) or SpxB to protect the cells from the oxidative toxicity (43, 81, 104). In the aerobic condition, the bacterium is shown to have higher *spxB* gene expression and more endogenous H_2O_2 production (81, 16, 96). The amount of acetyl-phosphate is also increased by the enzymatic process performed by the enzyme SpxB, which will be converted to ATP by the acetate kinase in the presence of acetate (81, 104).

O_2 + pyruvate + Pi ----------> CO_2 + H_2O_2 + acetyl-phosphate

SpxB-mediated colonization in pneumococcus

Colonization is required for invasion, transmission, and genetic manipulation of *S. pneumoniae* (42). The production of H_2O_2 by *S. pneumoniae* is suggested to have an inhibitory effect on its commensal flora (79). A knock-out mutation of *spxB* shows reduced virulence for nasopharyngeal colonization, pneumoniae, and bacteremia (7, 86, 99). The mechanism of *spxB* in colonization is not completely known. Phase variation has been shown to have an association with the H_2O_2 production by the bacterium; therefore, suggesting a larger network of pathways that may involve adaptation and the colonization of *S. pneumoniae*, and mutation of *spxB* has been shown to have a defect on the phase variants (80, 86). Maximum expression of *spxB* occurs during the early growth phase, and this corresponds with the time of pneumococcal competence (7, 62).

Modulation of cell competence by SpxB

Natural transformation is found in at least 40 bacterial species, and it is present in *S. pneumoniae* (66). Manipulation of DNA by this process has allowed the emergence of penicillin-resistant pneumococcal isolates and switching of capsules (22, 23, 76). Pneumococcal competence is induced by competence-stimulating peptide (CSP); consequently, it causes DNA uptake as well as DNA release (7, 70, 21). Activation of competence is a two-component signal transduction process, and the O_2 sensor ciaRH is a DNA-binding response regulator (4, 24, 37, 40). When O_2 is absent, this enzyme is thought to activate the protease HtrA and cleaves the CSP to down-regulate the

pneumococcal competence (24, 95). In addition, another O_2 sensor NADH oxidase (*nox*) is suggested to also play a role in the competence, and it optimizes the results of pneumococcal competence (4, 30, 116). The mechanism of cell lysis and the release of DNA due to the competence remains to be resolved. Mutation of the *spxB* reduces transformability and the ability of *S. pneumoniae* to release DNA, suggesting H_2O_2 is involved in the DNA release (7). Expressions of the competence-specific genes *comC* and *recA* are shown to have a reduced RNA level determined by real-time RT-PCR (7). In summary, factors that governing pneumococcal competence have been expanded to not only involve the enzymes CiaRH, HtrA, ComC, RecA, and Nox but also the enzyme SpxB. However, the mechanisms connecting *spxB* gene with competence remain to be explored.

H_2O_2 resistance by SpxB

During nasopharyngeal colonization, the amount of H_2O_2 production by *S. pneumoniae* may reach milimolar, and it is thought to confer an inhibitory effect on the competitive commensal flora (79). In addition, as mentioned before, H_2O_2 is thought to play a role in pneumococcal meningitis by inducing brain cell apoptosis (13). The mechanism by which *S. pneumoniae* resists the high amount of endogenous H_2O_2 is still not clearly known. Pericone et al. (81) showed that a *spxB*-deficient mutant exhibits reduced virulence for exogenous H_2O_2 resistance, and it is shown to have a correlation with H_2O_2 production. On the contrary, Rx1 cells pregrown in lactose are shown to have much higher resistance (at least 3-fold) to H_2O_2 challenged exogenously than any other

sugars tested (118). As mentioned before, there are many enzyme homologues, which serve to protect other bacteria from oxidative stress, that are also found in *S. pneumoniae*, but there is yet to be any experimental evidence that shows the function of the enzyme homologues. The NADH oxidase (*nox*) is also present in *Streptococcus mutans*. Its function is to protect the cells from oxidative toxicity, and it is shown to affect the transformability *S. pneumoniae* (29, 43). Attenuation of the *spxB* gene also affected the persistence of *S. pneumoniae* in the host.

Together, the pathway of pneumococcal competence is complicated, and it is regulated at least by CiaRH, HtrA, ComC, RecA, Nox, and SpxB. In addition, the role of *spxB* in H_2O_2 resistance is unclear, and determination of the relationship between H_2O_2 resistance and sugar metabolism is a pioneering work. The following studies were performed to investigate whether the lactose-regulated H_2O_2 resistance of *S. pneumoniae* has any effect on the induction of gene, and characterization of lactose-induced genes for H_2O_2 resistance was carried out. It is shown that in strain Rx1 (2), random mutagenesis of the chromosomal DNA showed induced gene expression as well as reduced H_2O_2 resistance in some of the isolates. This work was continued to determine the relationship between all the H_2O_2 resistance genes and the *spxB* by measuring levels of the H_2O_2 production. In addition, determination of other factors that regulate *spxB* activity was also carried out by measuring levels of H_2O_2 production.

CHAPTER III

METHODOLOGY

Bacterial strains and growth conditions.

Bacterial strains and plasmids used in the present study are listed in Table 1. To culture pneumococcal cells, casein hydrolysate (CAT) medium was used. It was prepared by dissolving 1% casein, 0.5% tryptone, 0.5% NaCl, and 0.1% yeast extract in one liter of distilled water, and sterilized by autoclaving. Unless otherwise stated, glucose and K_2HPO_4 were then added to yield a final concentration of 11 mM and 16 mM, respectively. This medium is now called CATPGlu. To make CAT agar media, 1.5% (W/V) of agar powder (DIFCO BiTek^{TM} AGAR) was added. Unless otherwise specified, pneumococcal cells were grown in a 13 mm slip cap tube using 1% of pneumococcal cells (at OD_{550} = 0.2) in CATPGlu broth, supplemented with a selective drug at suggested concentration demonstrated in Table 3, and incubated at 37ºC under ambient light. This provides a microaerobic growth condition. The growth of cells was monitored by measuring the turbidity with a spectrophotometer (Spectronic 20). CATP was used as the blank solution to obtain the actual reading of the turbidity. Pneumococcal cells grown to OD_{550} = 0.2 were supplemented with 10% sterile glycerol (V/V) in a 16 mm screw cap

tube to be stored at -80ºC. To study *spxB*, both microaerobic and aerobic conditions were used to grow in the flasks 125 ml, 250 ml, or 500 ml, which ever appropriate to contain the culture volume needed for the experiment. Aerobic growth conditions were obtained by using a shaker (New Brunswick Scientific) at 200 rotations per minute (r.p.m.). For the studies that required dark and aerated conditions, the window on the top of the shaker was covered with a piece of aluminum foil. Microaerobic and dark conditions were provided using a 37ºC incubator (VWR I530). Unless otherwise noted, pneumococcal cell density was assessed using the overlay method. Bacterial cells were transferred to 4 ml CATPGlu broth in a 16 mm cap tube containing 1/20 of 4% bovine serum albumin (BSA), vortexed to mix the contents, mixed with 4 ml of molten CATPGlu agar, kept in a 13 mm slip cap tube in the same 16 mm cap tube (this made the cell layer), and poured onto a CATPGlu agar – base layer, in a petri dish. Molten agar was kept warm at 42˚C to prevent solidification of CATPGlu agar. The buffer layer was made using 10 ml of molten CATPGlu agar poured on top of the cell layer. Unless otherwise specified, the drug layer was made using 10 ml of molten CATPGlu agar with an appropriate amount of specified drugs (Table 3) poured on top of the buffer layer. Plated cells were incubated microaerobically at 37˚C overnight (~20 hours) in a dark chamber.

TABLE 1. Bacterial Strains and Plasmids used

Strains	Genotype and/or relevant description	Source or reference
S. pneumoniae		
D39	Type 2	5
Rx 1	Type 2 unencapsulated	4
R6	Type 2 unencapsulated	106
CP1250	malM511 $str^{-}1$ β-gal^{-}	77
DP1004	$str^{-}1$	
SP1446	$Str^{-}1$ β-gal^{-} mal^{+} DP1004 x CP1250	
SP1541	*pyrB* Ω pHT19 derivative of CP1250 Em^{r} Str^{r}	This study
SP1542	*ccpA* Ω pHT20 derivative of CP1250 Em^{r} Str^{r}	This study
SP1543	*truB* Ω pHT16 derivative of CP1250 Em^{r} Str^{r}	This study
SP1545	*pyrB* Ω (pHT9 :: pEVP3) derivative of CP1250 Cm^{r} Spc^{r} Str^{r}	This study
SP1547	SP1541 carrying pHT9 for *pyrB* complementation Em^{r} Spc^{r} Str^{r}	This study
SP1548	SP1542 carrying pHT8 for *ccpA* complementation Em^{r} Spc^{r} Str^{r}	This study
SP1549	SP1543 carrying pHT10 for *truB* complementation Em^{r} Spc^{r} Str^{r}	This study
SP1551	*ccpA* Ω (pHT8 :: pEVP3) derivative of CP1250 Cm^{r} Spc^{r} Str^{r}	This study
SP1552	*truB* Ω (pHT14 :: pEVP3) derivative of CP1250 Amp^{s} Cm^{r} Str^{r}	This study
SP1559	*ccpA* Ω pHT20 (EcoRV) derivative of DP1004 Em^{r} Spc^{r} Str^{r}	This study
SP1564	SP1559 carrying pHT8 for *ccpA* complementation Em^{r} Spc^{r} Str^{r}	This study
SP1558	*pyrB* Ω pHT19 (*Eco*RV) derivative of DP1004 Em^{r} Spc^{r} Str^{r}	This study
SP1563	SP1558 carrying pHT9 for *pyrB* complementation Em^{r} Spc^{r} Str^{r}	This study
SP1552	*truB* Ω pHT14(*Bgl*II)::pEVP3 (*Bgl*II) derivative of CP1250 Amp^{s} Cm^{r} Str^{r}	This study
SP1566	*truB* Ω pHT16 (*Eco*RI) derivative of DP1004 Amp^{s} Em^{r} Str^{r}	This study
SP1565	SP1566 carrying pHT10 for *truB* complementation Em^{r} Spc^{r} Str^{r}	This study
SP1546	Spr1639 Ω pVJ618 derivative of CP1250 Cm^{r} Em^{r} Str^{r}	This study
SP1562	Spr1639 Ω pVJ618 (*Nco*I) derivative	This study

	of DP1004 Cm^r Em^r Str^r	
SP1572	*spxB* Ω pZH54 derivative of SP1446 Cm^r Str^r	This study
SP1612	SP1562 carrying pVJ619 for spr1639 complementation Em^r Spc^r Str^r	This study
SP1625	*spxB*+entire spr0643 Ω (3'spxB+entire spr0643::pEVP3) derivative of SP1446 Cm^r str^r	This study
SP1622	*spxB*+entire spr0643 Ω pHT27 derivative of SP1446 Em^r Str^r	This study
SP1623	*spxB*+entire spr0643 Ω pHT27 derivative of DP1004 Em^r Str^r	This study
E. coli		
XL1 blue MRA		
Stratagene		Stratagene
DH5α		
C600		

Plasmid	Genotype and/or relevant description	Source or reference
pACYC184	Cm^r Tc^r	18, 90
pUC8	derivative of pUC19 Amp^r	Stratagene
pUC18	derivative of pUC19 Amp^r	Stratagene
pDL278–D	Spc^r	27
pDG647	Amp^r Em^r derivative of pUC19	39
pEVP3	integrating plasmid in pneumococci carrying promoterless *lacZ* gene Cm^r	20
pVJ609	pACYC184(EcoRV)::1.2 kb PCR fragment (using the primers VJ123-F & VJ124-R) carrying pneumococcal spr1639 that codes for catabolite-control protein Cm^r Tc^s	
pVJ611	pSK+(*Hin*dIII)::1.6 kb(*Hi*ndIII) fragment containing Em^r cassette of pDG647	
pVJ617	pVJ609 (*Sal*I) the largest fragment self-ligated	
pVJ618	pVJ617(*Eco*RV)::1.6 kb(*Sma*I) Em^r cassette of pVJ611 (Inserted within spr1639) Cm^r Em^r	
pVJ619	Spc^r, lac^- pDL278(*Sma*I) :: 1.85 kb (*Cla*I) Klenowed fragment of pVJ617, carries spr1639	
pVJ577	Em^r pRL425(*Hin*cII)::klenowed Em^r cassette from pDG647	
pZH54		
pHT8	pDL278::1.5 kb(*Sma*I) PCR fragment (using the primers, VJ125-F & VJ126-R) carrying pneumococcal *ccpA* (spr1813) gene that codes for catabolite-control protein Spc^r	This study
pHT9	pDL278::1.44 kb(*Sma*I) PCR fragment (using the primers, VJ131-F & VJ132-R) carrying pneumococcal *pyrB* (spr1155) gene that codes for aspartate carbamyl transferase Spc^r	This study
pHT10	pDL278::1.1 kb (*Sma*I) PCR fragment (using the primers, VJ129-F & VJ130-R) carrying pneumococcal *truB* (spr1092) gene that codes for tRNA pseudouridine synthase Spc^r	This study

pHT14	pUC8::1.1 kb(*Sma*I) PCR fragment (using the primers, VJ129-F & VJ130-R) carrying pneumococcal *truB* (spr1092) gene that codes for tRNA pseudouridine synthase Amp^r	This study
pHT16	pHT14::1.6 kb fragment carrying the Em^r determinant from pDG647 within the *truB* gene Amp^r Em^r	This study
pHT19	pHT9::1.6 kb fragment carrying the Em^r determinant from pVJ577 (originally derived from pDG647) within the *pyrB* gene Em^r Spc^r	This study
pHT20	pHT8::1.6 kb fragment carrying the Em^r determinant from pVJ577 (originally derived from pDG647) within the *ccpA* gene Em^r Spc^r	This study
pHT26	pUC18::0.8 kb (*Sma*I) PCR fragment (using primers VJ171-F & VJ172-R) from the mRNA of DP1004 pneumococcal strain, carrying the 3' end of the *spxB* and the entire spr0643 Amp^r	This study
pHT27	pHT26(*Xmn*I)::1.6 kb fragment carrying the Em^r determinant from pVJ611 (originally derived from pDG647)(*Xmn*I) within the spr0643 gene Amp^r Em^r	This study
pHT28	pEVP3::0.8 kb(*Sma*I) PCR fragment (using primers, VJ171-F & VJ172-R) from the mRNA of DP1004 pneumococcal strain, carrying the 3' end of the *spxB* and the entire spr0643. This was derived from SP1625 pop-out plasmid Cm^r	This study

TABLE 2. Primers used in this study.

Primer pairs	Sequence	Description*
pEVP3-F	5’-CTTCCACAGTAGTTCACCACCT-3’	Forward primer for pEVP*3*
pEVP3-R	5’-ACCCGGGAGCTCGAATTCTA-3’	Reverse primer for pEVP3
VJ115	5’-ATTCGGCGGCTCAATCGGGG -3’	Forward primer for internal *spxB*
VJ116	5’-GATACCAGGAAGGGCAATAC-3’	Reverse primer for internal *spxB*
VJ123	5’-ACTTTTTTGAAGGGGAAG-3’	Forward primer for spr1639
VJ124	5’-ATAGAAACTGAATGGAGGC-3’	Reverse primer for spr1639
VJ125	5’-CTAAGGTGGTAGCAAGGAGC-3’	Forward primer for *ccpA*
VJ126	5’-CGGACTAACAGGACAGGC-3’	Reverse primer for *ccpA*
VJ127	5'-CGATTTATTTGAAGGACGCAC-3'	Forward primer for *trmE*
VJ128	5'-TACCTCTTGTTCCAGCACC-3'	Reverse primer for *trmE*
VJ129	5’-ATTGTCCTCCTCCAGAG-3’	Forward primer for *truB*
VJ130	5’-ATTGTTATCTATCACCCG-3’	Reverse primer for *truB*
VJ131	5’-TGGATACTAAACCTTTCCGTG-3’	Forward primer for *pyrB*
VJ132	5’-GTAATGGATTCTTGGTAGCCG-3’	Reverse primer for *pyrB*
VJ159	5’-ATGACTCAAGGGAAAATTACTGCA-3’	Forward primer for *spxB*
VJ160	5’-TTATTTAATTGCGCGTGATTGCAATCC TTCTTCTTC-3’	Reverse primer for *spxB*
VJ171	5’-GGTGTAGACTTCACAAACGCTG-3’	Forward primer for 3’*spxB*+entire spr0643
VJ172	5’-GACCTATTTCATACGATAAAAATCAAG-3’	Reverse primer for 3’*spxB*+entire spr0643

*Unless otherwise specified, these primers will amplify the entire genes.

TABLE 3. Antibiotic drugs used in this study.

	Escherichia coli		*Streptococcus pneumoniae*	
Drugs	**Broth conc.**	**Overlay conc.**	**Broth conc.**	**Overlay conc.**
Ampicilin	**75**	**75**	**25**	**25**
Chloramphenicol	**15**	**15**	**5**	**15**
Erythromycin	**1000**	**1000**	**1**	**1**
Spectinomycin	**350**	**350**	**150**	**150**
Streptomycin	**Not used**	**Not used**	**200**	**200**
Tetracycline	**10**	**10**	**10**	**10**

Construction of mutants.

Transformation of *E. coli* and *S. pneumoniae* was performed as described previously (41, 6). Genomic DNA from Rx1 (4) was extracted and purified as described in a previous method (68). DNA fragments containing the open reading frames of *spxB*, spr0643 – 3'*spxB*, spr1813, spr1639, *truB*, and *pyrB*, and *trmE* were amplified from Rx1 chromosomal DNA with the respective primers listed in table 2 by using a PCR machine (Eppendorf Mastercycler) and the PCR fragments were purified as described by Maniatis et al (67). Gel-purifird PCR fragments were ligated to different plasmid vectors for the respective purposes as follow: pEVP3 (gene transcriptional studies) (20), pDL278 (gene complementational studies) (27), pUC8/pUC18/pUC19 (Stragagene)/pACYC184 (18, 90)/pSK+ (gene knock-out studies). Restriction sites of every gene and the plasmid carriers are listed in table 1. As shown in table 1, an erythromycin cassette (39) was inserted within every gene carried by the plasmid vectors. The resulting recombinant plasmids were proliferated in *E. coli* cells (Table 1) and introduced into the strains of Rx1 as illustrated in table 1. The transformants were selected on CATPGlu agar plates containing erythromycin (1 μg/ml). Inserts were confirmed by sequencing carried out at the OSU core facility. Knock-out of spr1813, spr1639, *pyrB*, and *truB* were confirmed phenotypically by a lower survival rate (3-fold) than the wild-type after challenged with 10 mM H_2O_2 (Wal-mart). The accession number for each each: *spxB* NP_358236, spr1639 NP_359231, spr1813 NP_359405, *pyrB* NP_358748, *trmE* NP_358514, *truB* NP_358685.

Experimental assays.

A. β-galactosidase assays

This was performed to quantify an expression of a gene in pneumococcal cells with the suggested growth condition mentioned above. Unless specified otherwise, the cells were harvested at OD_{550} = 0.3. To carry out this assay, 1 ml of cells in a 13 mm slip cap tube was kept on ice for 10 min to slow down cell growth. The cells were mixed with 50 µl of lysis solution [10% (v/v) triton-X-100 with 0.04% DOC (w/v) in distilled water], vortexed, and lysis was performed at 37˚C for 5 min or until the solution became clear. To start the β-galactosidase reaction, 0.5 ml lysate was transferred into a 1.5 ml disposable cuvette containing 0.5 ml of Z buffer (1.61 g of $Na_2HPO_47H_2O$, 0.55 g of $NaH_2PO_4H_2O$, 0.075 g of KCl, 0.025 g of $MgSO_47H_2O$ in 100 ml distilled water, autoclaved, 0.27 ml of β-mercaptoethanol added before use), and 0.2 ml of ONPG (10 mg/ml). The reaction was carried out in the dark at room temperature for 90 min or until yellow color was developed. To stop the reaction, 0.5 ml of Na_2CO_3 (1 M) was added to the reaction mixture, mixed using the same pipette tip by pipetting up and down 3 times. Then, the optical density at 420 nm was measured in a spectrophotometer (Spectronic 1001, Milton Roy CO.). Miller units were calculated by using the formula: Miller unit = 1000 * OD_{420} /time of reaction (minute) /OD_{550} (cell density) (67).

B. H_2O_2 sensitivity assays

Bacterial cells, pregrown to OD_{550} = 0.1 were diluted by 10^3-fold before distributing 1 ml aliquots of the diluted sample into four test tubes, and three of the diluted samples (one diluted sample was the control for no H_2O_2 treatment) treated with 10 mM H_2O_2 (3% original, from Wal-mart) were incubated with the control at 37ºC for 30 min. H_2O_2 challenged samples were then mixed with 4 ml of CATPGlu broth containing 4% of sheep blood (provided by the college of Veterinary Medicine at OSU) to remove exogenous H_2O_2 and all tubes were kept on ice until plating. Cell density with or without H_2O_2 treatment was assessed on agar plates by the overlay method. The survival rate in percent was assessed by dividing the CFU of 1 ml cultures after exposure to H_2O_2 by the CFU of the control tube without H_2O_2.

C. H_2O_2 production assays

This was performed to measure the amount of H_2O_2 being released by the cells. No cell lysis was carried out. A stardard plot was established to correlate amount of H_2O_2 (mM) to OD_{560} absorption value (Fig. 2). 1 ml of each culture was spun down at 15,000 x g in 1.5 ml centrifuge tubes for 5 min at room temperature (~25˚C). 200 µl aliquots of each supernatant was mixed with 700 µl of CATP, and 100 µl of a solution containing 1.5 mg of ABTS [2,2'azinobis(3-ethylbenzthiazolinesulfonic acid)]/ml and 0.1 mg of horseradish peroxidase/ml in 0.1 M potassium phosphate buffer (pH 7.0). Blank solution was prepared using 700 µl CATP, 200 µl spin-down CATPGlu, and 100 µl substrate. The

reaction was allowed to take place at room temperature under dark condition for 15 min. Optical density at OD_{560} was measured and result was recorded. The formula, y = 0.4159x – 0.1118 for calculating H_2O_2 concentration in mM was deduced from the standard curve demonstrated in figure 2.

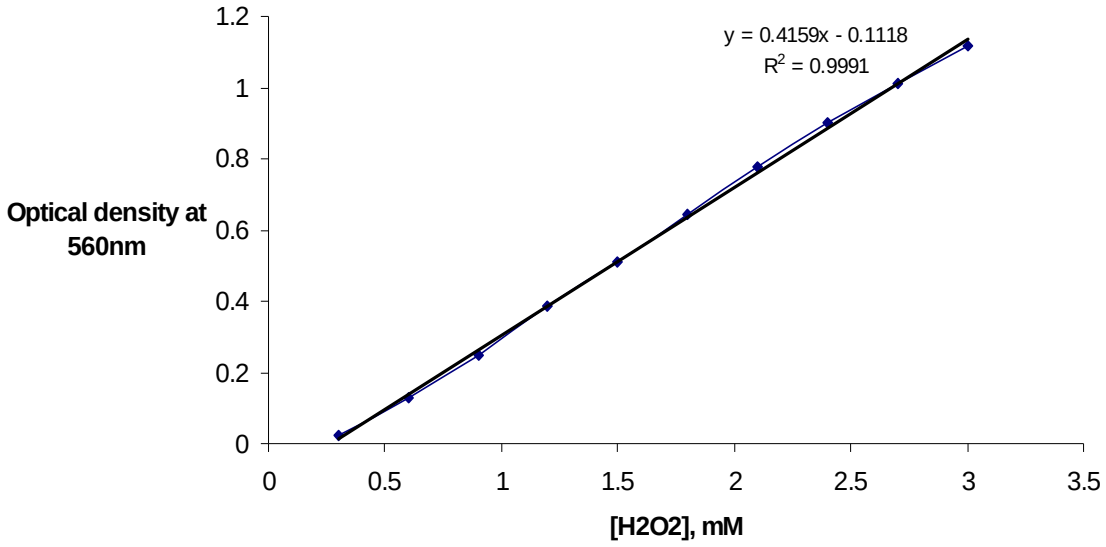


Figure 2. Standard plot for the measurement of H_2O_2 concentration (mM). Reaction was done with 0.1 ml of H_2O_2.

Search for the presence of an extra-cellular peptide inducing H_2O_2 production.

5 ml aliquots of bacterial cells at OD_{550} = 0.3 grown in 125 ml flask were distributed into seven sterile 125 ml flasks containing 1/100 volume of supernatant harvested from pregrown cells at different growth phases, and all the samples including a control (added sterile distilled water, no supernatant added) were incubated under ceiling lights for 90 min. H_2O_2 production assay was then performed for each tube.

Another experiment was performed using proteinase K (20 mg/ml) mixed with 5 ml aliquots of bacterial cells pregrown to OD_{550} = 0.23 in three of 125 ml flasks, and another three of the same flask containing the same culture treated with sterile distilled water were used as controls, followed by incubation of each sample at 37˚C under ceiling lights with aeration. Optical density at OD_{550} of each sample was measured. H_2O_2 production assay was then performed for each tube.

CHAPTER IV

FINDINGS

Part I. Resistance to exogenous H_2O_2 in *S. pneumoniae*.

(a) Identification of genes which are up-regulated during growth in lactose.

The growth of pneumococcal cells in lactose led to significantly increased survival when exposed to exogenous H_2O_2. We sought to identify the genes that are not involved in the metabolism of lactose but which confer the observed resistance to oxidative stress. To accomplish this goal, the pneumococcal chromosome was randomly mutagenized by the insertion-duplication. The insertion-duplication mutagenesis involves insertion of a heterologous reporter gene within the chromosome via transformation. This is done first by ligating a fragment of chromosomal DNA to the heterologous DNA *in vitro*. The ligated circular DNA is introduced into the cells by transformation. The DNA taken up circularizes inside the cell and the chromosomal fragment of the DNA directs the insertion of the heterologous reporter DNA at the site of homology. During this process, the directing DNA fragment is directly duplicated flanking the heterologous DNA by a mechanism that is still unclear (Fig.3) (63). The reporter gene we employed

in this study was the heterologous *E. coli* plasmid vector, pEVP3 (20). This plasmid carries *E. coli* origin of replication and is incapable of independent of replication in pneumococcal cells. It carries a chloramphenicol resistance gene that expresses both in *E. coli* and pneumococcus. We only found three mutants that were blue on CATPLac and X-gal but remained white on CATPGlu and X-gal plates. The level of expression of genes from these three mutants was measured by β-galactosidase assay (Fig. 5, 6).

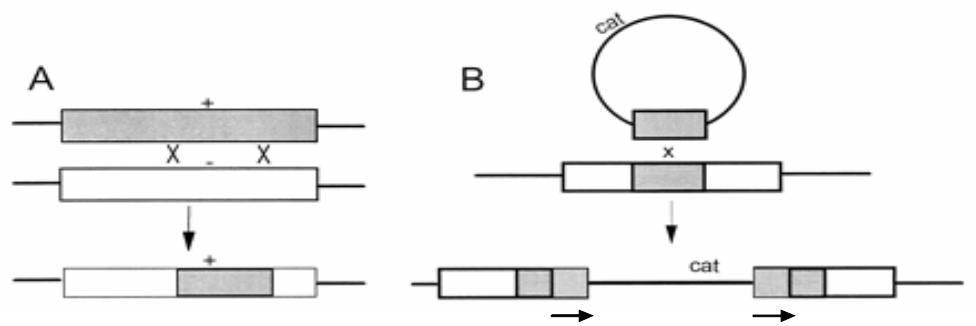


Figure 3. Mechanism of insertion-duplication in *S. pneumoniae*. Contrasting ways of recombination which depend upon the topology of the donor DNA in transformation of pneumococcus. Panel A shows the integration of a genetic marker by double recombination involving the flanking DNA. Heterologous marker in a circular DNA can be inserted with the help of the directing DNA that gets duplicated as direct repeats as shown in panel B.

Next, I sought to identify the place of insertion of the reporter plasmid in each of these clones. For this purpose, I grew each of the clones without any selective pressure for several generations overnight at 37^{o}C. Due to the presence of the generation of direct repeats in the insertion-duplication mutagenesis employed in the construction of these mutants, it was expected that the direct repeats would recombine with each other leading to the excision of the inserted DNA. Such a spontaneous "curing" has been reported to occur in such mutants at a low but detectable frequency (20, 63). The excised circular molecule would be unable to replicate and be expected to be eventually lost. Following overnight growth, chromosomal DNA was isolated from these cultures. The purified

chromosomal DNA was used in transformation of *E. coli* cells. Due to the presence of *E. coli* origin of replication, the "cured" plasmid molecules were able to yield transformants. The sequence of passenger DNAs in the *E. coli* recombinant plasmids isolated from each of these clones was subsequently obtained using the primers, pEVP3-F and pEVP3-R. The DNA sequence determination showed that the three genes which were up-regulated by growth in lactose were *bgaA* (spr0565), in pHT4, *pyrB* (spr1155), in pHT6, *trmE* (spr0920), in pHT7, and *truB* (spr1092), in pHT5. Orfs of *pyrB* and *truB* were amplified as described in Methods and Materials. We did not pursue our study with the *bgaA* gene because it has been well studied and the encoded function β-galactosidase has been confirmed (56, 117). Later, we were interested in the role of pneumococcal catabolite control proteins (CCP), spr1639 and spr1813. Both spr1813 and spr1639 have been listed in the family of lactose repressive protein, *LacI* (NCBI). However, only spr1813 (CcpA) has been experimentally shown to possess the regulatory function (38, 50, 56). An implication that the spr1813 regulates the transcription of *pyrB* has been reported recently (44). It is also shown to regulate the *bgaA* operon by binding to the catabolite-responsive element (*cre*) site located adjacent to the promoter (56) (Fig. 4). There is no *cre* site on the operons of *trmE* and *truB* that have been determined by comparing the *cre* sequence to the sites around the promoters. However, no report on spr1639 function is available to date. So, we also amplified the orf of these two *ccp* genes. In summary, mutations were introduced in the genes spr1639, spr1813, *pyrB*, and *truB*. Introduction of mutation into *trmE* gene was not successful due to cloning of the gene in *E. coli* did not produce any true clone.

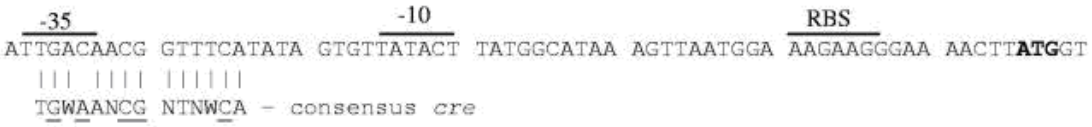


Figure 4. Promoter region of the *pts-bgaA* operon. The *cre* consensus sequence is aligned below the promoter sequence (54).

(b) Identification of mutants susceptible to H_2O_2

We then determined whether lactose-induced genes were responsible for H_2O_2 resistance. Lactose pregrown cultures were exposed to H_2O_2, and survival was assessed by colony counts. Strains SP1541, SP1543, SP1558, SP1559, SP1562, SP1566, carrying an erythromycin cassette inserted within the genes *pyrB*, *truB*, spr1813 or spr1639, were found to have lost resistance to H_2O_2 by at least 3-fold lower than the wild-type, as determined by fold difference of the survival rates (survival in lactose divided by survival in glucose) even when pregrown in lactose (Table 4, 5). Mutation of *pyrB* gene both in CP1250 and DP1004 backgrounds was found to have no significant differences in the survival rates when compared with the wild-type.

(c) Complementation studies

We next determined whether loss of the genes contributed to the increased H_2O_2 sensitivity. Strains SP1547, SP1549, SP1563, SP1565, complementing to mutagenized genes were constructed by introducing a recombinant plasmid of pDL278 carrying a respective gene, under control of the plasmid promoter, into the mutants.

Complementation of *truB*- and spr1813-deficient mutants in DP1004 background restored H_2O_2 resistance. In fact, H_2O_2 resistance was significantly higher in *pyrB*-complemented mutant (SP1558) than its parent strain. The insertion-mutagenesis that inactivates *pyrB* in the SP1541 and SP1558 strains is likely to have a polar effect, since *pyrB* operon has been shown to carry downstream genes of *carA* and *carB* in a close bacterial species *Lactobacillus plantarum* (72). Arrangement of genes by NCBI also supports this finding (Fig. 26). The resulting *carA* and *carB* are unable to produce peroxynitrite, a highly cytotoxic compound, resulting higher survival to H_2O_2 (Fig. 26, 27) (44, 71). However, failure of SP1541 to restore resistance to H_2O_2 could be due to a mutation in β-galactosidase gene in CP1250 which contributed to the polar effect of the mutant. On the other hand, significant low resistance of spr1639-complemented mutant to H_2O_2 might have caused by the gene that being highly expressed from the plasmid.

In summary, I have (a) identified 5-genes in pneumococcus, *truB, trmE, pyrB, ccpA*, spr1639, that are upregulated during growth in lactose as compared to in glucose, (b) shown that these are not involved in the metabolism of lactose, and (c) that at least 3-genes, *truB, pyrB*, and *ccpA*, which are involved in conferring 3-fold increased survivability to H_2O_2 when grown in lactose. The loss of the gene activities resulted in the decreased survival whereas complementation restored the effect. The specific mechanism employed by the genes that regulates the resistance to H_2O_2 is unknown.

TABLE 4. Percent survival rate of the mutants and the complemented strains created following H_2O_2[(b)] treatment, in CP1250 background (10^4 CFU)

		Grown in		
Strains	Genotype	Glucose[(a)]	Lactose[(a)]	Fold difference
SP1543*	*truB*$^-$	1.7±1.6	2.5±1.9	1.5
SP1549[§]	*truB* complement	3.0±2.4	4.0±2.9	1.3
SP1541*	*pyrB*$^-$	0.6±0.3	2.5±1.6	4.2
SP1547[§]	*pyrB* complement	1.9±2.3	4.8±3.7	2.5
CP1250	Wild-type	2.3±1.1	13.4±7.0	5.7

(a) Concentration of glucose used = 11 mM, concentration of lactose = 11 mM.

(b) Concentration of H_2O_2 used for treatment = 10 mM. Time of H_2O_2 exposure = 30 min. Mean values of triplicates from six independent experiments (±SE) are presented. * Negative mutant P values: SP1543: $P < 0.05$, SP1541: $P > 0.05$. [§] Complement P values: SP1549: $P > 0.05$, SP1547: $P > 0.05$.

Table 5. Percent survival rate of the mutants and the complemented strains created following H_2O_2[(b)] treatment, in DP1004 background (10^4 CFU)

		Grown in		
Strains	Genotype	Glucose[(a)]	Lactose[(a)]	Fold difference
SP1566$^{\Theta}$	*truB*$^-$	5.0±4.0	3.1±1.7	~1.0
SP1565$^{\Delta}$	*truB* complement	2.2±1.0	6.4±2.7	~2.9
SP1558$^{\Theta}$	*pyrB*$^-$	3.7±1.7	6.7±1.9	~1.8
SP1563$^{\Delta}$	*pyrB* complement	6.3±1.9	67.0±12.0	~6.7
SP1559$^{\Theta}$	spr1813$^-$	48.0±13.0	27.0±7.0	~1.0
SP1564$^{\Delta}$	spr1813 complement	3.3±0.5	10.0±0	~3.0
SP1562$^{\Theta}$	spr1639$^-$	1.8±0.8	1.0±0	~1.0
SP1612$^{\Delta}$	spr1639 complement	1.4±0.4	1.6±0.26	~1.0
DP1004	Wild-type	4.2±0.3	13.0±2.6	3.0

(a) Concentration of glucose used = 11 mM, concentration of lactose = 11 mM.
(b) Concentration of H_2O_2 used for treatment = 10 mM. Time of H_2O_2 exposure = 30 min. Mean values of triplicates from six independent experiments (±SE) are presented. $^{\Theta}$ Mutant P values: SP1559/SP1562/SP1566: $P < 0.05$, SP1558: $P > 0.05$. $^{\Delta}$ Complement P values: SP1565: $P > 0.05$, SP1563: $P < 0.005$, SP1564: $P > 0.05$, SP1612: $P < 0.05$.

(d) Gene expression studies.

In order to quantify expression of the genes, we carried out β-galactosidase assays for strains SP1545 (*pyrB*), SP1546 (spr1639), SP1551 (spr1813), SP1552 (*truB*), constructed by inserting a *lacZ* gene adjacent to the promoters of the genes, in the presence of glucose or lactose. Unexpectedly, different levels of expression were observed only in *truB* and spr1639, and both demonstrated approximately 4-fold higher expression in lactose than glucose (Fig. 5, 6) (Table 6).

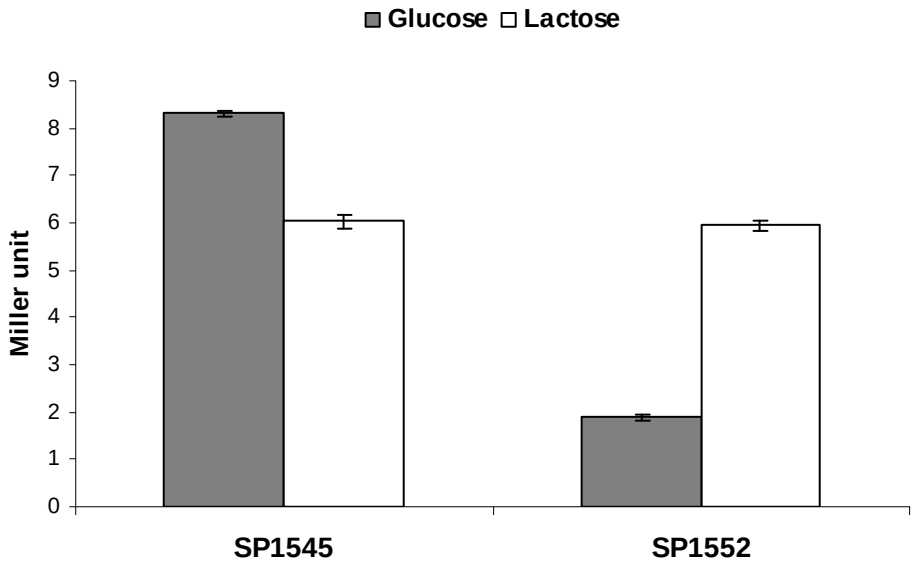


Figure 5. Transcriptional levels of the genes *pyrB* in SP1545, and *truB* in SP1552. These strains were constructed by insertion-duplication within each of the gene and promoterless *lacZ* was located downstream of the promoter of each gene. The cells were grown microaerobically in glucose and lactose, separately at 37ºC. Cells at turbidity OD_{550} = 0.3 were harvested for β-galactosidase measurement, using Miller unit. Each mutant was derived from CP1250. CP1250 carries knock-out genes in beta-galactosidase and also maltose metabolism. Unless specified or otherwise, β-galactosidase expression levels in CP1250 were observed at ~ 0.3 in glucose and 0 in lactose miller units. Representative results of the average of one independent experiment in triplicate are shown. N = 3.

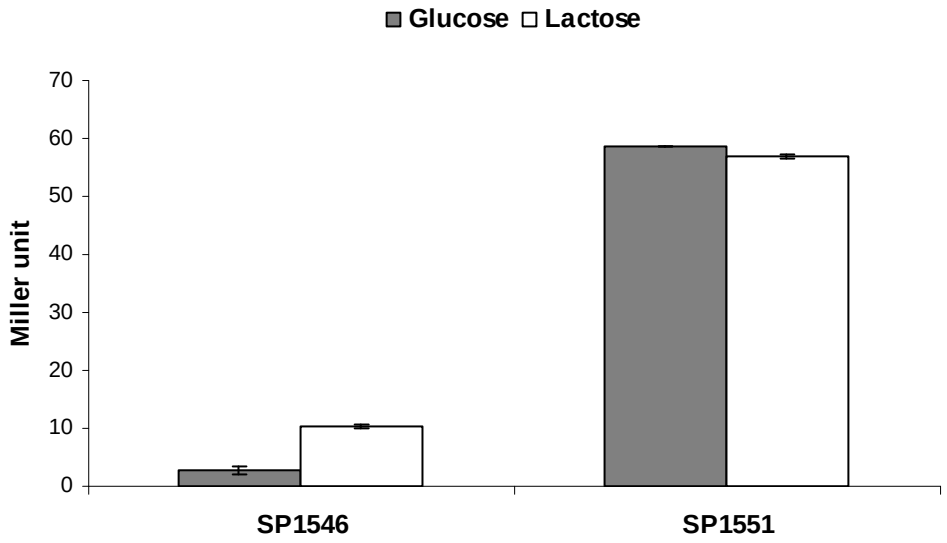


Figure 6. Transcriptional levels of the genes spr1639 in SP1546 and spr1813 in SP1551. This strain was constructed by insertion-duplication within the gene and promoterless *lacZ* was located downstream of the promoter. The cells were grown microaerobically in glucose and lactose, separately at 37ºC. At OD_{550} = 0.3, the cells were harvested for beta-galactosidase measurement, using Miller unit. SP1546, *mal*$^{+}$, *lac*$^{-}$, was derived from CP1250 homologously recombined with DP1004 DNA. Representative results of the average of at least 1 independent experiment in triplicate are shown. N = 3.

(e) Cultural characteristics of the mutants.

We then determined whether the mutants have any defect in the general phenotypic properties by conventional tests and measurement of the growth rates. Results of the conventional tests were tabulated in Table 6. Physiologically, the growth rates were found to behave similar to wild-type, but *pyrB*-deficient mutant, *pyrB*-complemented mutant, and spr1813-complemented mutant, which failed to have the cell density exhibited by the wild-type (Fig. 7). Two independent growth curves were performed, and both showed the same doubling times for each pair of mutant.

Mutation of *pyr*B inactivates a mechanism required for arginine and pyrimidine biosynthesis; therefore, it contributed to a defect in the growth rate of SP1558 and SP1563. Low cell density of SP1564 might have been caused by the gene being highly expressed from the plasmid, and abundance of it might repress growth-related genes. Overall, the data suggested that there was no significant defect in general phenotypic characteristics, but some growth rates were affected.

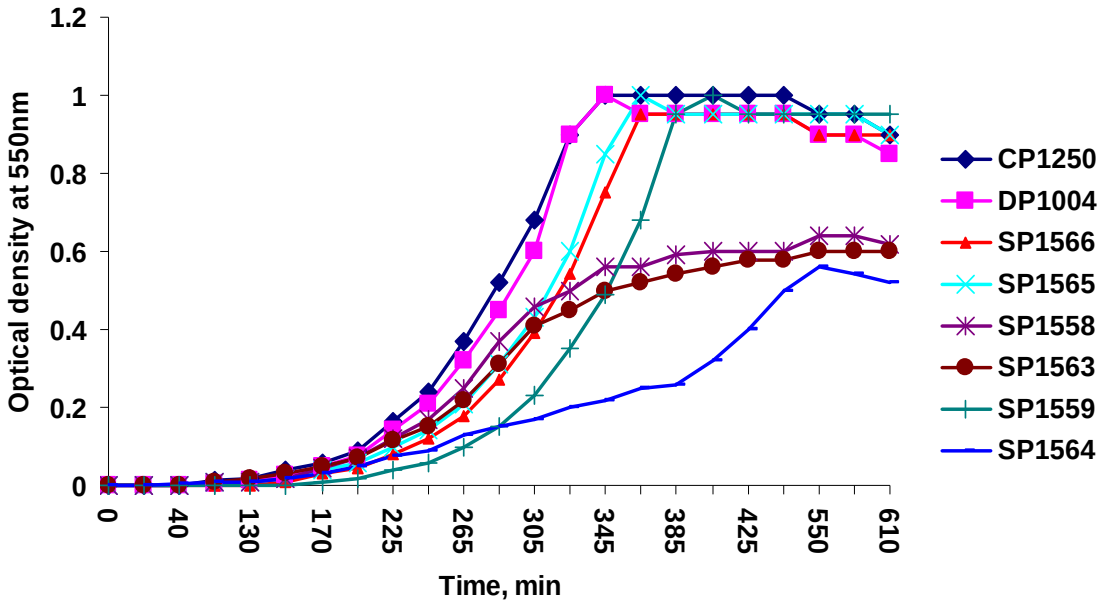


Figure 7. Growth curves of cells carrying a mutation in the lactose-regulated genes. These genes have been tested for exogenous H_2O_2 resistance both in glucose and lactose. Mutagenized form of these genes decreases its resistance to exogenous H_2O_2. This result was established by growing these strains microaerobically in CATPGlu in 13 mm slip cap test tubes at 37ºC. Cell growth was monitored by measuring turbidity at OD_{550}. Representative results of one independent experiment are shown.

Table 6: Overall cultural characteristics of spr1639, spr1813, *pyrB* and *truB* mutants.

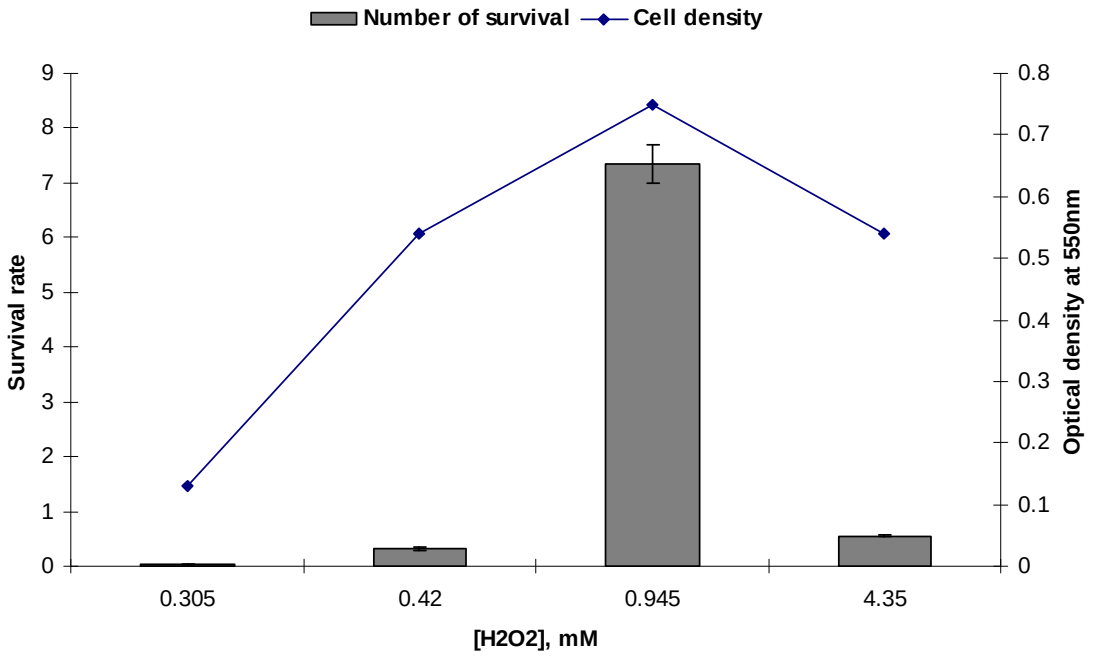
Strains/gene	Cell Morph.	Hemolytic pattern	Bile solubility	Autolysis	B-gal assay, Miller unit	H_2O_2 Sensitivity Lac/glu (ratio)
SP1551	Gram "+", diplococci	α-hemolytic	Yes	Yes	Glu (58.5) Lac (57.0)	-
SP1559/spr1813$^-$	Gram"+", diplococci	α-hemolytic	Yes	Yes	-	~1.0
SP1564/spr1813 complement	Gram "+", diplococci	α-hemolytic	Yes	Yes	-	~3.0
SP1545	Gram "+", diplococci	α-hemolytic	Yes	Yes	Glu (8.30) Lac (6.00)	-
SP1558/*pyrB*$^-$	Gram "+", diplococci	α-hemolytic	Yes	Yes	-	~1.0
SP1563/*pyrB* complement	Gram "+", diplococci	α-hemolytic	Yes	Yes	-	~6.7
SP1552	Gram "+", diplococci	α-hemolytic	Yes	Yes	Glu (1.89) Lac (5.96)	-
SP1566/*truB*$^-$	Gram "+", diplococci	α-hemolytic	Yes	Yes	-	~1.0
SP1565/*truB* complement	Gram "+", diplococci	α-hemolytic	Yes	Yes	-	~3.0
SP1546	Gram "+" diplococci	α-hemolytic	Yes	Yes	Glu (2.30)	
SP1562/spr1639$^-$	Gram "+" diplococci	α-hemolytic	Yes	Yes	Lac (10.4)	~1.0
SP1612/spr1639 complement	Gram "+" diplococci	α-hemolytic	Yes	Yes	-	~1.0

Part II: H_2O_2 resistance is mediated by SpxB activity

(a) H_2O_2 resistance is correlated with H_2O_2 production.

In a previous study, the concentration of H_2O_2, determined from the supernatant of cultures grown microaerobically in glucose, was found to be maximal during stationary phase (7). This finding suggested that levels of resistance might also vary throughout the growth phases (81). The H_2O_2 production was determined simultaneously with H_2O_2 sensitivity test from cultures grown microaerobically to early log-phase, mid-log phase, stationary phase and death phase. As expected, the culture producing increased concentration of H_2O_2 also showed more resistance to H_2O_2 (Fig. 8A). In addition, the culture with no elevated H_2O_2 production was also found to have an increased H_2O_2 resistance as the growth phase entering stationary phase (Fig. 8B). Similar result was observed in another experiment (data not shown). Together, the data suggested that there was an association between H_2O_2 resistance and the growth phase.

A



B

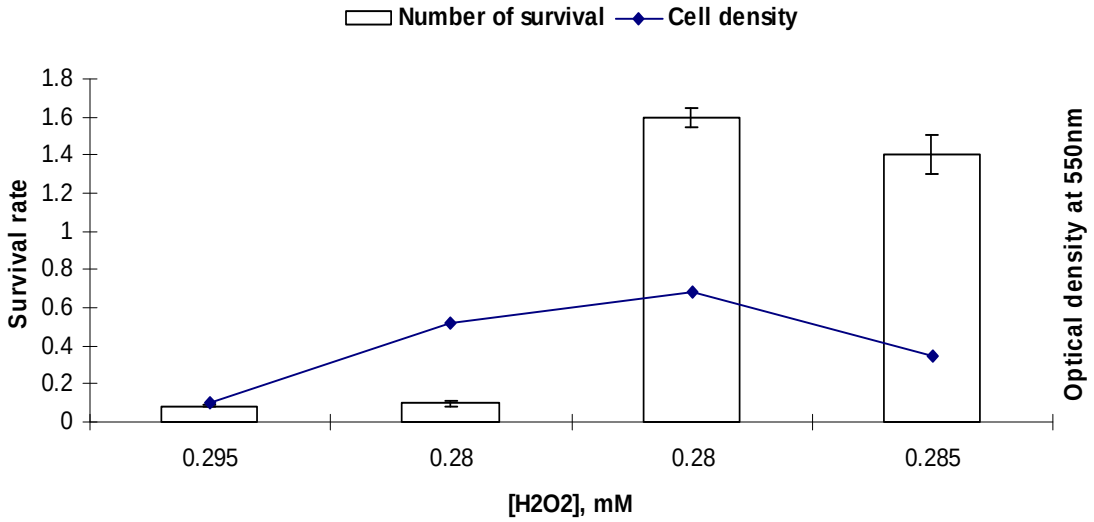


Figure 8. Effect of exogenous H_2O_2 on the survival rate of DP1004, grown to different phases of growth. Cells were grown microaerobically in CATPGlu at 37ºC. Cells from various growth phases were treated with 10 mM exogenous H_2O_2. Simultaneously, endogenous H_2O_2 production of the cells and its cell density were measured before the exogenous H_2O_2 treatment. Bars represent number of survivors per 1000 cells while the line represents cell density measured at the various points. X-axis represents accumulated H_2O_2 concentration, mM harvested at various growth points, the left Y-axis represents survival rate, and the right Y-axis represents cell density OD_{550}. DP1004 was used for this study. It is derived from Rx1 pneumococcal strain. (A) Pneumococcal cells grown in a 250 ml flask. (B) Pneumococcal cells grown in a 13 mm diameter of 5 ml tube. Mean values of duplicates from one independent experiment (±SE) are presented. N = 2.

(b) Monitoring *spxB* expression during growth.

We next tested the hypothesis that *spxB* expression would also be maximal during stationary phase. The transcriptional levels of the gene *spxB*-null mutation in SP1572, constructed by inserting a *lacZ* gene adjacent to the promoter of *spxB*, at various growth points were measured. Unexpectedly, the transcriptional level of *spxB* during stationary phase was minimal, while its maximum transcription was found at early- as well as mid-log phases (Fig. 8, 11). Similar expression profile was observed in another two experiments (data not shown).

In order to determine whether the incorrelation of *spxB* expression with the H_2O_2 production was due to an artifact, we measured both simultaneously. Strain 1625 was constructed as demonstrated in figure 10 because previous construction of SP1604 (contains only the *spxB* orf) exhibited no H_2O_2 production (data not shown). This newly made strain allowed us to measure both spxB expression and the H_2O_2 production, so a clean correlation could be established. In SP1604, two copies of entire *spxB* orfs are expected due to the insertion-duplication by pEVP3 recombinant plasmid which causes genome shifting and the loss of ability to produce H_2O_2 (Fig. 9).

Figure 9. Suggested arrangement of *spxB* and the genes surrouding it, NCBI (69).

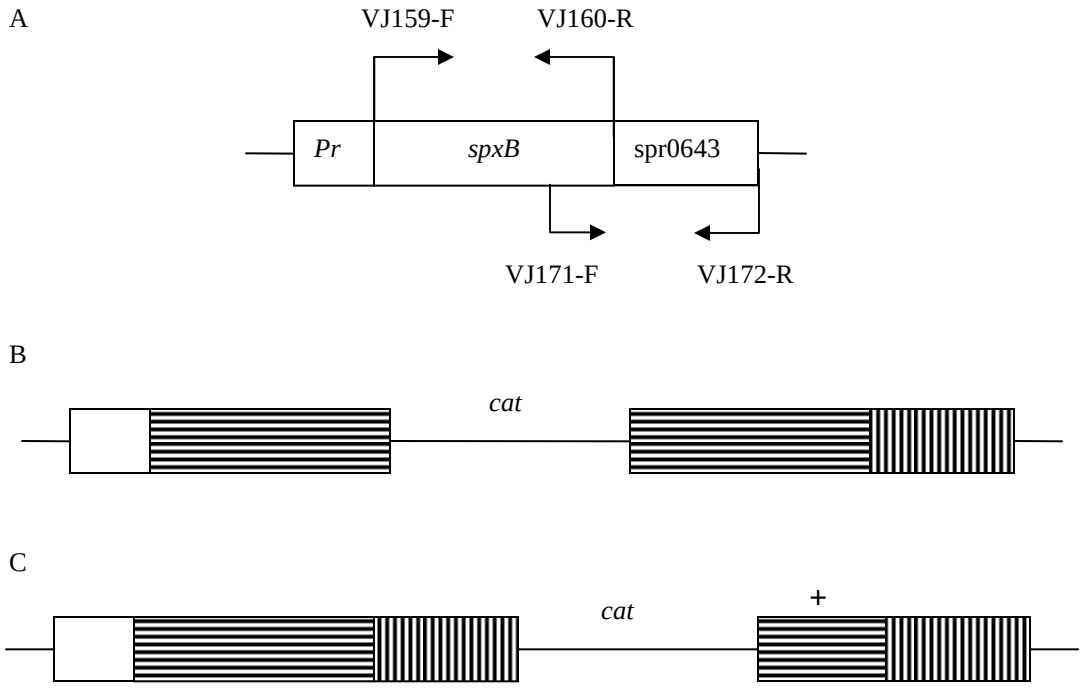


Figure 10. Illustration of recombination products from circular chimeric DNA donors targeting the *spxB* operon. (A) Suggested arrangement of *spxB* and the glyoxalase (spr0643) gene, obtained from NCBI. Primers sequences are available in table 2. (B) A recombination product resulting from the duplication of entire *spxB* gene carried in pEVP3 plasmid, pZH54, generated by ligating PCR product to the plasmid. (C) A recombination product resulting from the duplication of 3' end *spxB* which is represented by (+) through the whole orf of glyoxalase. (B) and (C): the horizontal-striped boxes represent the region of *spxB* gene, the vertical-striped boxes represent the region of glyoxalase, and the white box represent the promoter of the operon.

Both levels of *spxB* expression, measured by β-galactosidase assay, and H_2O_2 production were measured from unwashed and washed samples. Samples were washed to avoid any artifact that might be caused by accumulation of H_2O_2 during growth. In both cases, the observations were consistent with previous findings demonstrating that there was no parallel relationship between expression of *spxB* and the activity of the enzyme (Fig. 8, 11, 12). A similar result was also observed in another experiment (data not shown).

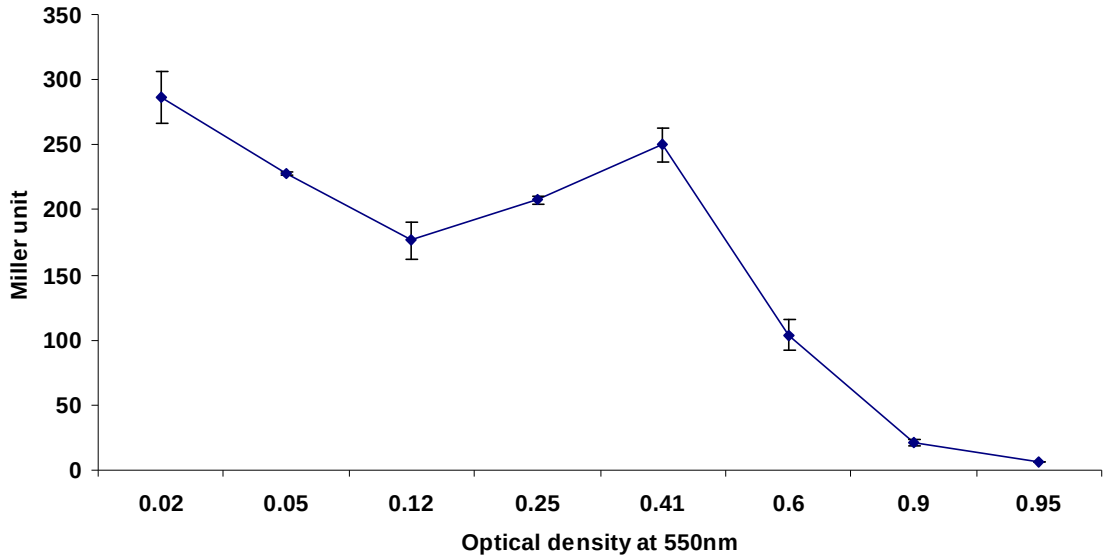
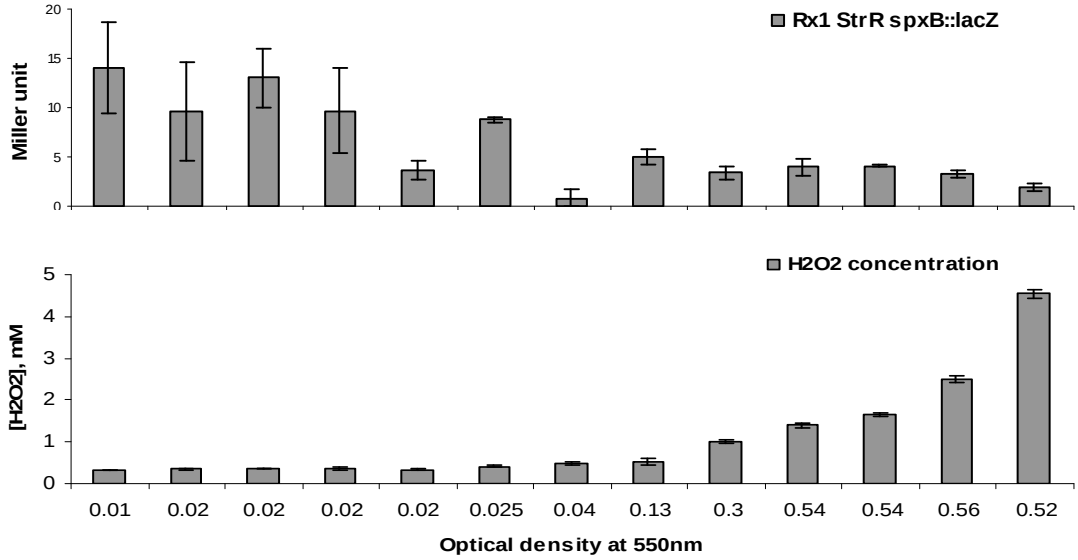


Figure 11. Transcriptional level of *spxB* during the growth cycle. To study this, a strain was constructed by insertion duplication within the gene and promoterless *lacZ* was located downstream of its promoter; namely, SP1572. The cells were grown microaerobically in glucose at 37 ºC in a 125 ml flask. Every 40 minutes of incubation, the cells were harvested for β-galactosidase measurement, using the Miller unit. SP1572 was derived from SP1446. Mean values of duplicates from one independent experiment (±SE) are presented. N = 2.

A.



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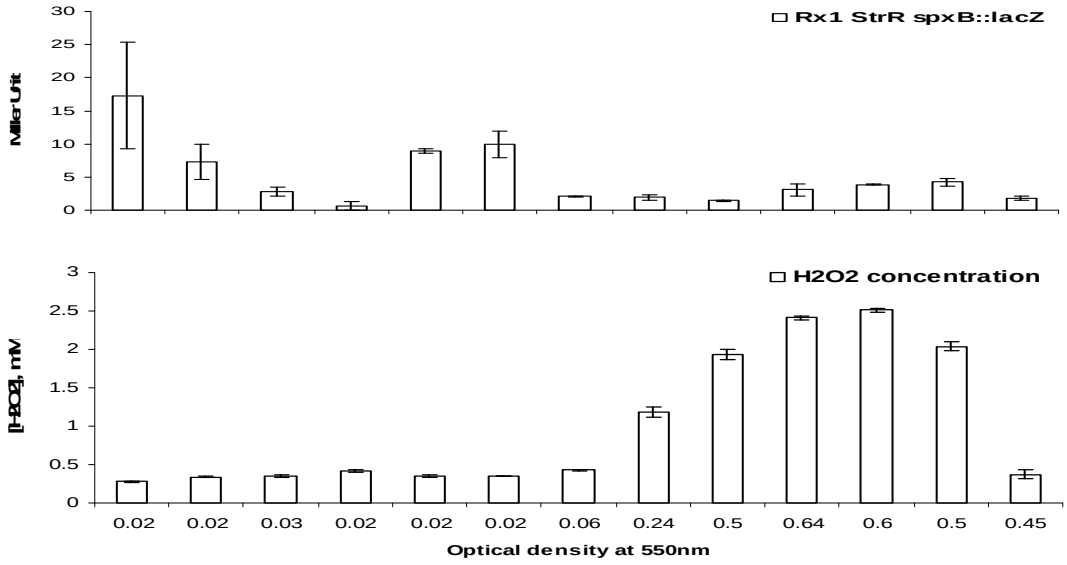


Figure 12. Study of SpxB activity in line with its expression from sample, SP1625: before washed/unwashed (A), and washed (B). Cells were grown microaerobically at 37˚C in CATPGlu in a 250 ml flask. OD_{550} was monitored and 6 ml cells were harvested at OD_{550} = 0.02 with 1 ml used for β-galactosidase assay of *spxB* expression and the endogenous H_2O_2 measurement, and the rest was washed in fresh CATP broth before resuspended in 5 ml fresh CATPGlu containing Cm^r for selection. 1 ml of resuspended cells was used for H_2O_2 measurement (background, no significant detection; data not included here) and the rest was incubated for 1 hour at the same condition used before. The result is demonstrated in figure (B). Mean values of duplicates from one independent experiment (±SE) are presented. N = 2.

(c) Function of spr0643 in H_2O_2 production.

In a previous report, *spxB* was found to come from a monocistronic operon (86). We therefore tested the hypothesis that spr0643 is not part of *spxB* operon. cDNA was prepared by using primers that are able to provide the transcript of 3' *spxB* – entire spr0643 (total size of ~800 bases) if *spxB* and spr0643 were co-transcripted. The expected size of cDNA was present in a gel picture (Fig. 13). Sequence result of the cDNA suggested that the spr0643 was copresent with *spxB* on the same transcript, and spxB operon was not monocistronic.

Then, we determined whether unexpressed spr0643 was responsible for the loss of ability to produce H_2O_2. The spr0643 knock-out mutants were also constructed in *lac*-deficient background of *S. pneumoniae* strain to avoid any artifact from the removal of β-galactosidase activity. The H_2O_2 concentratons present in overnight cultures SP1622 and SP1623 were found to be similar as in the wild-type strains (Fig. 14). This suggested that there was no direct involvement of spr0643 in the H_2O_2 production, and the SP1604 may not be a true clone.

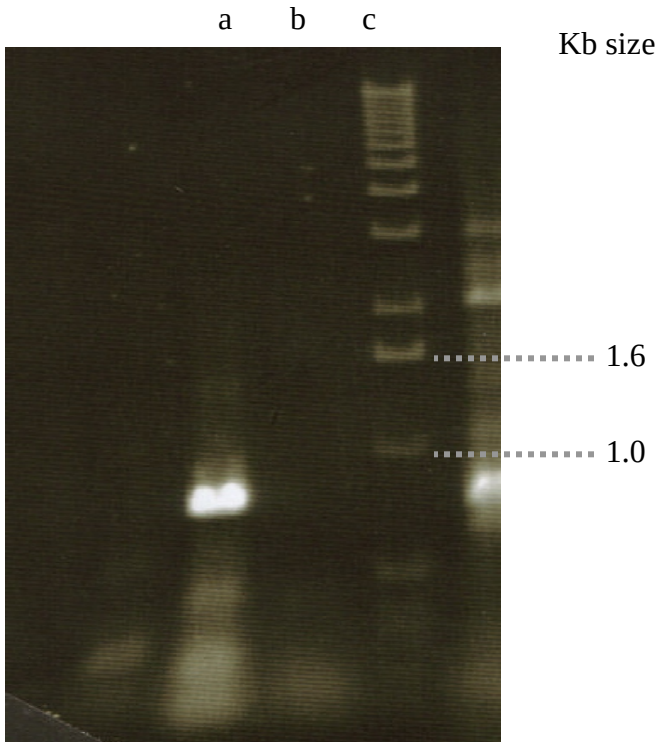


Figure 13. Agarose gel electrophoresis of cDNA resulting from the product of RT-PCR of DP1004 using primers VJ171-F and VJ172-R. (a) cDNA produced from DNA with primers VJ171-F and VJ172-R. (b) DNA Negative control by treating RNA sample with RNase. (c) Size markers are given by 1 kb ladder.

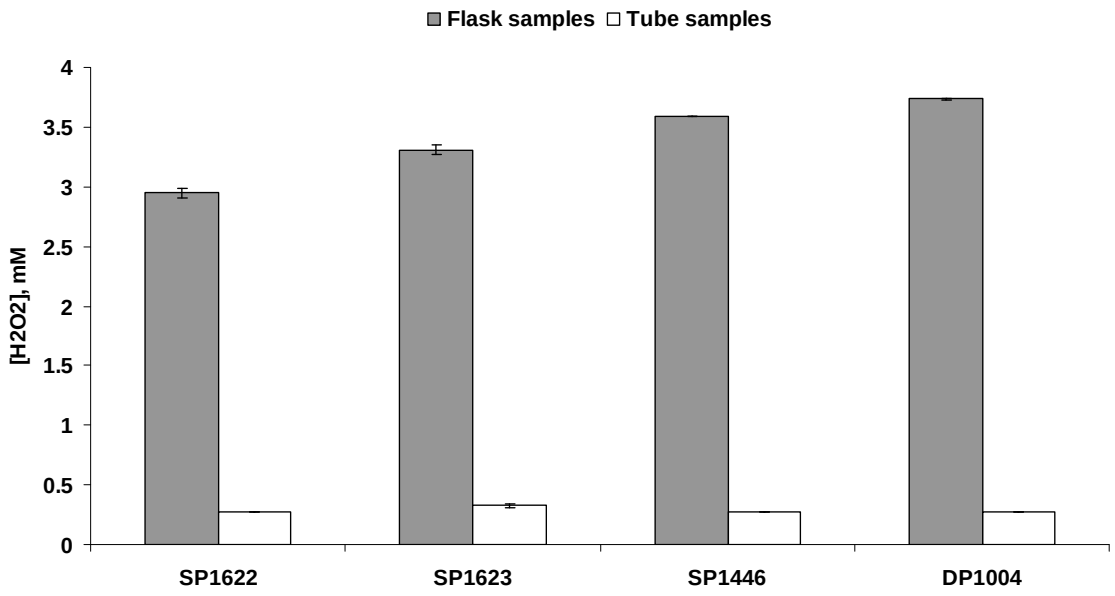


Figure 14. Measurement of H_2O_2 produced by glyoxalase-deficient mutants. Ability of glyoxalase-deficient mutants to produce H_2O_2 was studied by measuring H_2O_2 amount from overnight culture grown microaerobically at 37˚C under ambient light. Mutants are SP1622 (glyoxalase mutant in SP1446 background), and SP1623 (glyoxalase mutant in DP1004 background). The SP1446 is genotypically the same as in the DP1004 and CP1250 except, it is β-galactosidase negative and maltose metabolism normal, respectively. Cells were grown both in flasks and tubes. The supernatant was harvested from sample after about 20 hours incubation for H_2O_2 measurement. Mean values of duplicates from one independent experiment (±SE) are presented. N = 2.

(d) Monitoring H_2O_2 production for the non-catabolic mutants.

Previously, mutants carrying a mutation in the genes spr1639, spr1813, *pyrB* or *truB* were highly sensitive to H_2O_2 (Table 4, 5). We therefore tested the hypothesis that strains that were sensitive to H_2O_2 would also be unable to produce H_2O_2. The H_2O_2 concentrations from overnight cultures were determined. Lacose was also used because it seemed to induce H_2O_2 resistance through these genes (Table 4, 5). Cultures grown in glucose showed reduced H_2O_2 concentrantration in mutants SP1558 (*pyrB*-deficient, 2-fold lesser), SP1559 (spr1813-deficient, 5-fold lesser), and SP1566 (*truB*-deficient, 5-fold lesser) while no apparent defect was found in the mutant SP1562 (spr1639-deficient) even when grown in the presence of lactose (Fig. 15).

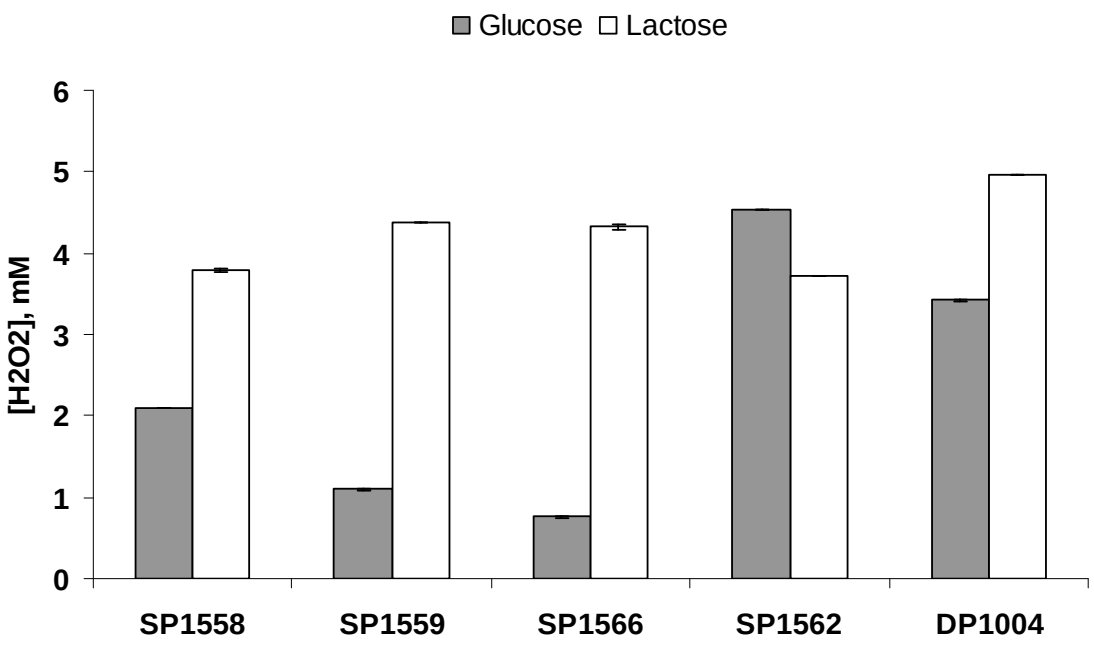


Figure 15. Measurement of H_2O_2 produced by spr1639, spr1813, *pyrB*, *truB* mutant strains. These negative mutants carry a gene that is responsible for catabolite-regulated H_2O_2 resistance. They are SP1558 (*pyrB*$^-$), SP1559 (spr1813$^-$), SP1566 (*truB*$^-$), and SP1562 (spr1639$^-$). These insertion mutants are derived from DP1004 background, the wild-type of this test. Cells were grown microaerobically in glucose and lactose, separately at 37˚C, in a separate 125 ml flask, under ambient light. Supernatant was harvested from overnight samples after 20 hours incubation for H_2O_2 measurement. Mean values of duplicates from one independent experiment (± SE) are presented. N = 2.

(e) Monitoring H_2O_2 production during growth in different carbohydrates.

In a previous study, H_2O_2 resistance of pneumococcal cells was found to be highly reduced when grown in several sugars, but in lactose (Table 1). We therefore determined whether loss of the resistance was due to a reduction in the H_2O_2 production. The H_2O_2 concentrations from overnight cultures were determined. Normal H_2O_2 concentrations (3 – 5 mM) were found in all supernatants of cultures grown in all types of sugar except, in fructose or galactose which most likely due to the cultures did not grow well in those sugars (Fig. 16).

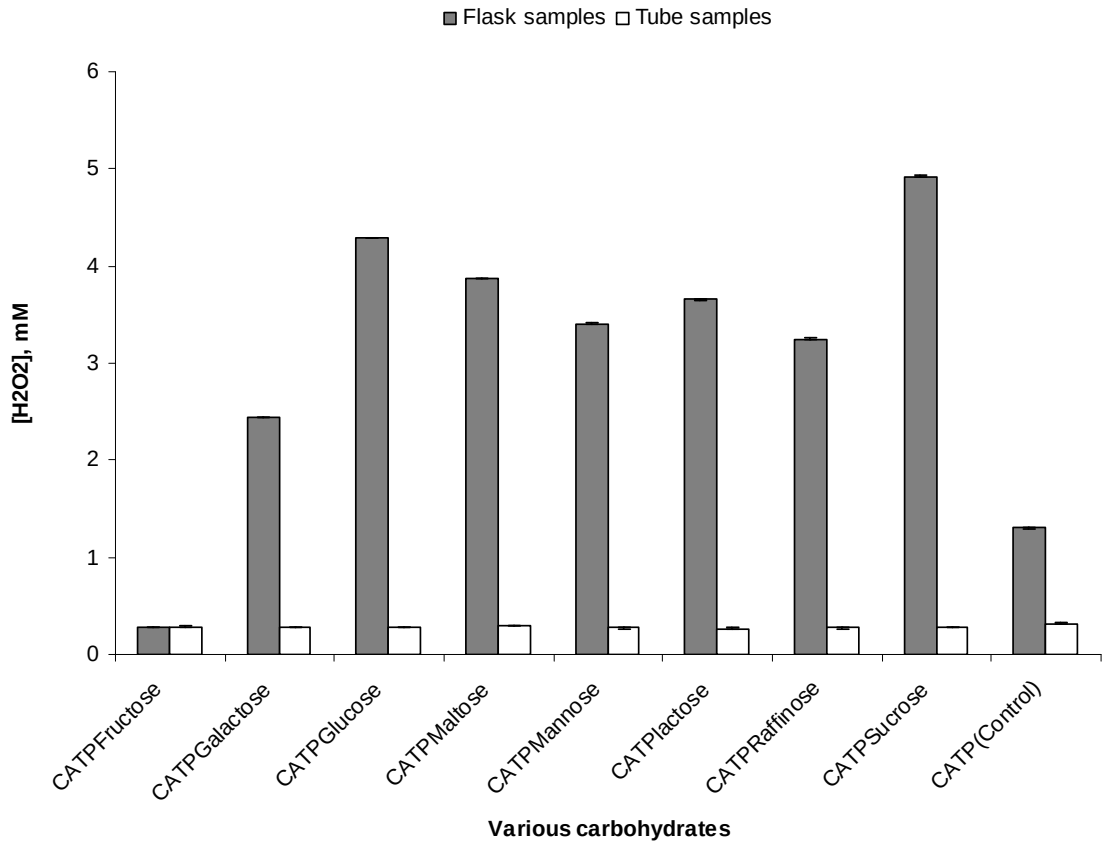


Figure 16. Measurement of H_2O_2 produced by DP1004 grown in various carbohydrates. DP1004 cells were grown microaerobically at 37˚C in various sugars, separately in a 125 ml flasks and a 13 mm slip cap test tube. Both the tubes and the flasks were incubated overnight under ambient light. Supernatants were harvested from sample after about 20 hours incubation for H_2O_2 measurement. The DP1004 is the wild-type strain. Mean values of duplicates from one independent experiment (±SE) are presented. N = 2.

In order to determine whether highly reduced H_2O_2 concentrations in cells grown in fructose or galactose were due to inability to produce H_2O_2, we monitored growth rates in those sugars. Cultures were also grown in other sugars for comparison purpose. Turbidities were monitored at various growth points until death phases were reached. Growth curves were plotted for demonstration purpose. A similar doubling time at 40 minutes was found among cultures grown in all tested sugars, but galactose (~80 minutes) (Fig. 17). Cell density was found to be 2-fold less with the culture grown in fructose compared with others. This suggested that there was an association between growth ability and the *spxB* activity.

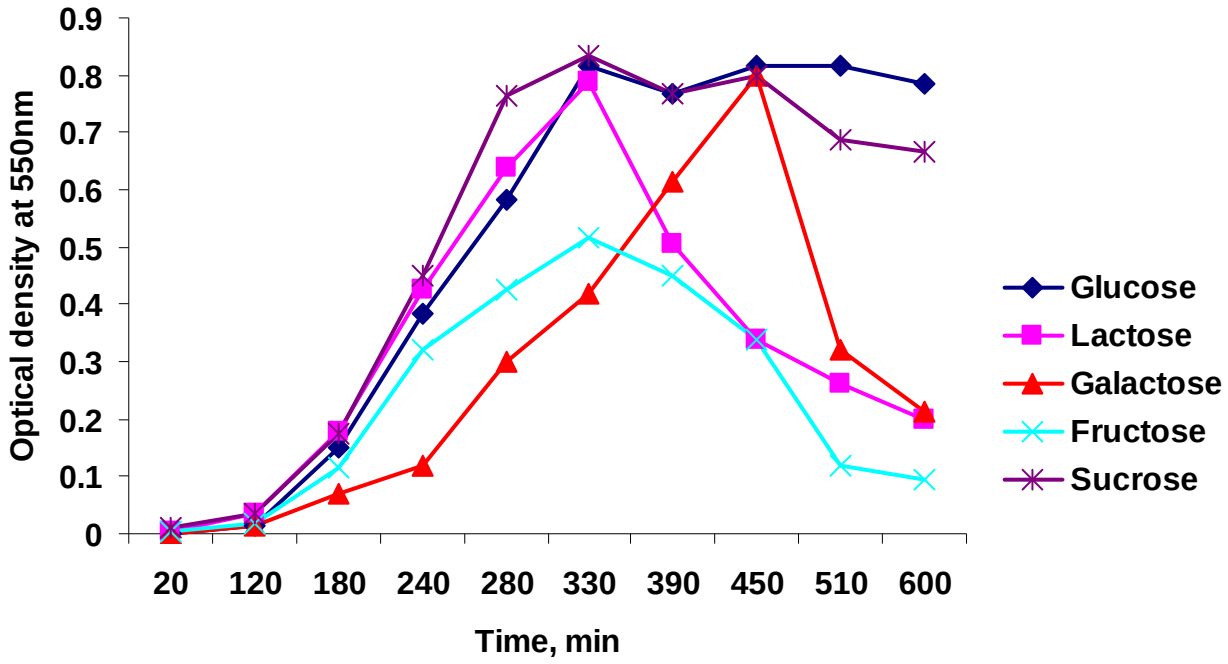


Figure 17. Growth curves comparison from pneumococcal cells grown in various carbohydrates. DP1004 cells were grown microaerobically at 37˚C in different carbohydrates in 13 mm slip cap test tubes, under ambient light. Cell density was monitored by measuring turbidity at OD_{550} throughout the life cycle. The DP1004 is the wild-type strain. Representative results of one independent experiment are shown.

We then checked whether higher H_2O_2 resistance in cultures pregrown in lactose to OD_{550}~0.1 was due to induced H_2O_2 production. The H_2O_2 concentration present in supernatants of cultures was determined. As expected, at turbidity 0.1, about 2-fold higher H_2O_2 concentration was found in the supernatant containing lactose than the glucose (Fig. 18, 19A). In fact, the H_2O_2 production was found to gradually increase when grown in lactose while gradual increment in glucose was observed only during stationary phase. Together, the data suggested that lactose-mediated H_2O_2 resistance requires the presence of SpxB, but H_2O_2 production could be eliminated.

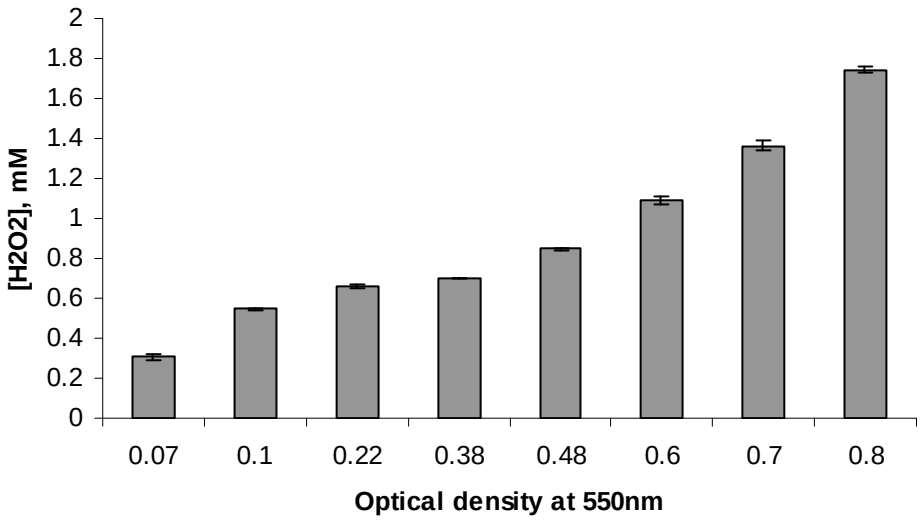
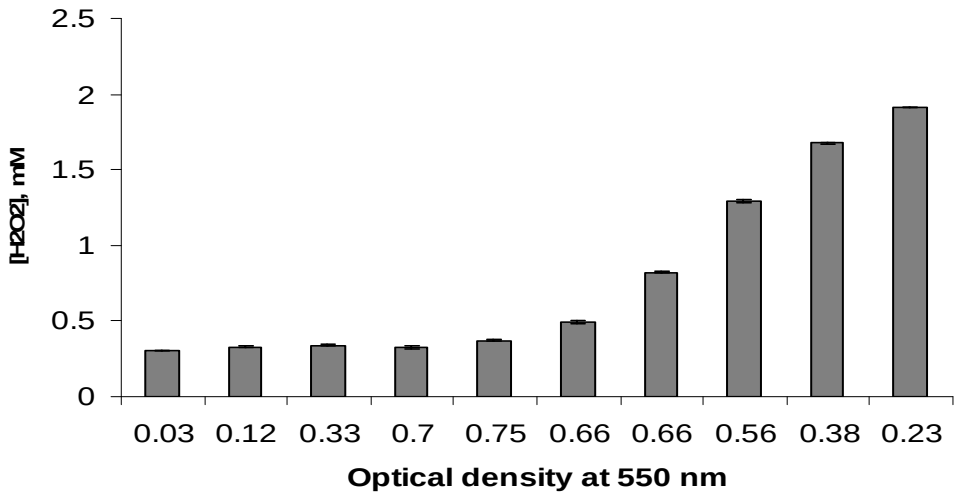


Figure 18. Production and accumulation of H_2O_2 during the growth cycle. Cells were grown microaerobically in lactose, at 37˚C, under ambient light. OD_{550} was measured throughout the life cycle and H_2O_2 measurement was done every 40 minutes of growth, started from OD_{550} = 0.07. The DP1004 is the wild-type strain. Mean values of duplicates from one independent experiment (±SE) are presented. N = 2.

In a previous report, glucose was found to repress H_2O_2 production in *S. pneumoniae* (104). We therefore tested the hypothesis that transferring H_2O_2 producing cells to fresh medium would repress H_2O_2 production. The H_2O_2 concentration present in supernatants of unwashed or washed cultures was determined. Cell density from washed samples was assessed by colony counts. In contrast, H_2O_2 production was found to continue in washed cultures originated from stationary phase which contained highest H_2O_2 concentration as well as cell density (Fig. 19). A similar observation was found in previous study using SP1625 to correlate spxB expression with the activity of washed cultures (Fig. 12B).

In order to determine whether high cell density was responsible for induced H_2O_2 production, we measured H_2O_2 concentration present in supernatants of diluted cultures originally pregrown to stationary phase. H_2O_2 production was found to continue in all diluted samples (Fig. 20). Together, the data suggested that H_2O_2 production could be induced by high cell density and was inreversible after commitment.

A.



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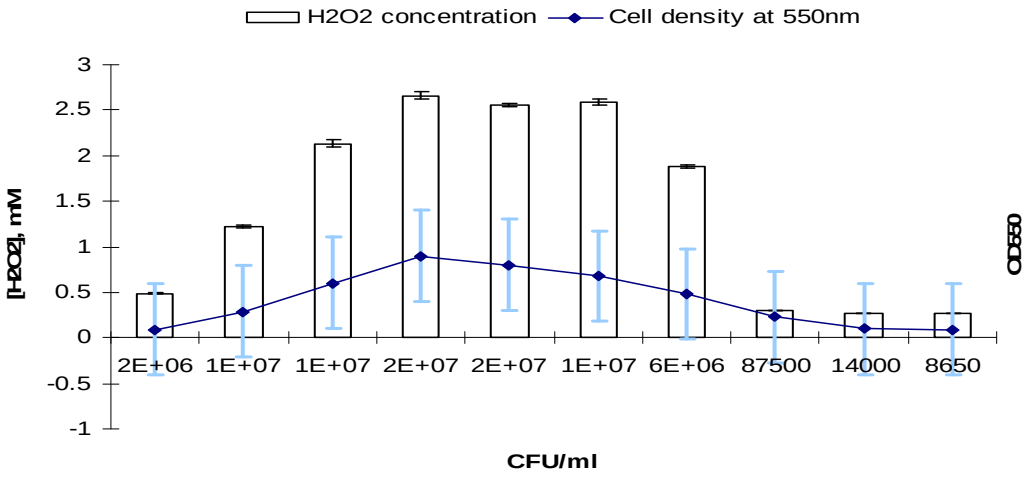


Figure 19. Regulation of the ability of DP1004 to produce H_2O_2 by the growth phase and cell densitiy. (A) Growth phase changes the ability of DP1004, grown in CATPGlu to produce H_2O_2. Cells were grown microaerobically in CATPGlu in a 250 ml flask, at 37˚C, under ambient light. Cell density was monitored throughout the cell life cycle, started from OD_{550} = 0.03. Supernatant was harvested at every 1 hour of incubation for accumulated H_2O_2 measurement. This is the result of unwashed sample. Cell density was monitored by measuring turbidity at OD_{550}, as demonstrated on x-axis. Subsequently, H_2O_2 was measured and demonstrated on Y-axis (gray bars). Result from washed sample is in (B). (B) Cell density changes the ability of DP1004 to produce H_2O_2. After cells were washed at every one hour incubation, cells were resuspended in fresh CATPGlu and grown microaerobically in a 125 ml flask, at 37˚C, under ambient light for one hour. Cell density was monitored by measuring turbidity at OD_{550} (Line with dots, right-Y-axis) and 50 µl cells was stored at -80˚C in 10% glycerol for viable plating later (white bars, X-axis). 1/20 dilution was introduced to the sample during storage. 1 ml of the culture was used to carry out H_2O_2 measurement assay (white bars, left-Y-axis). Mean values of duplicates from one independent experiment (±SE) are presented. N = 2.

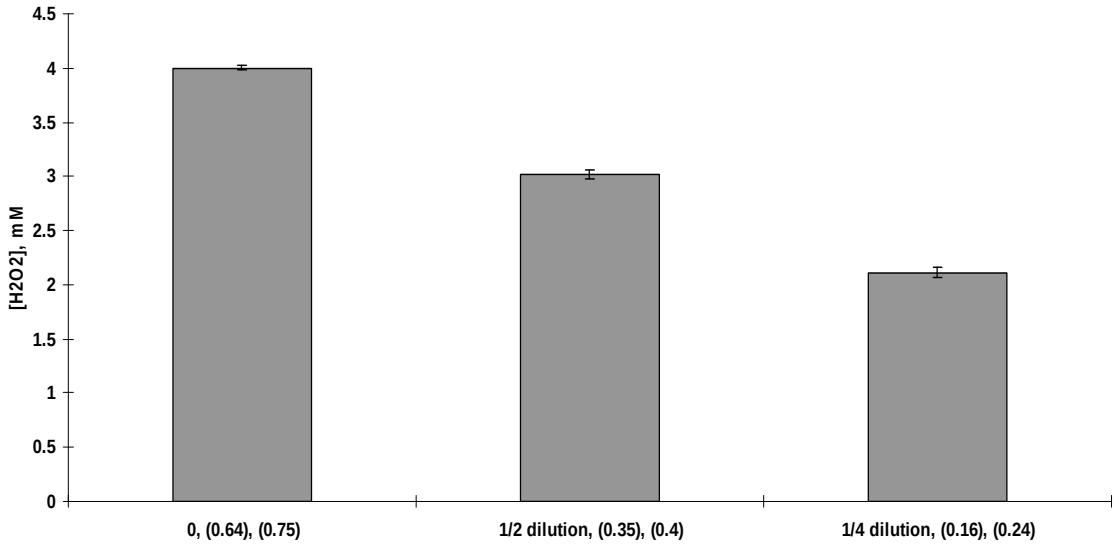


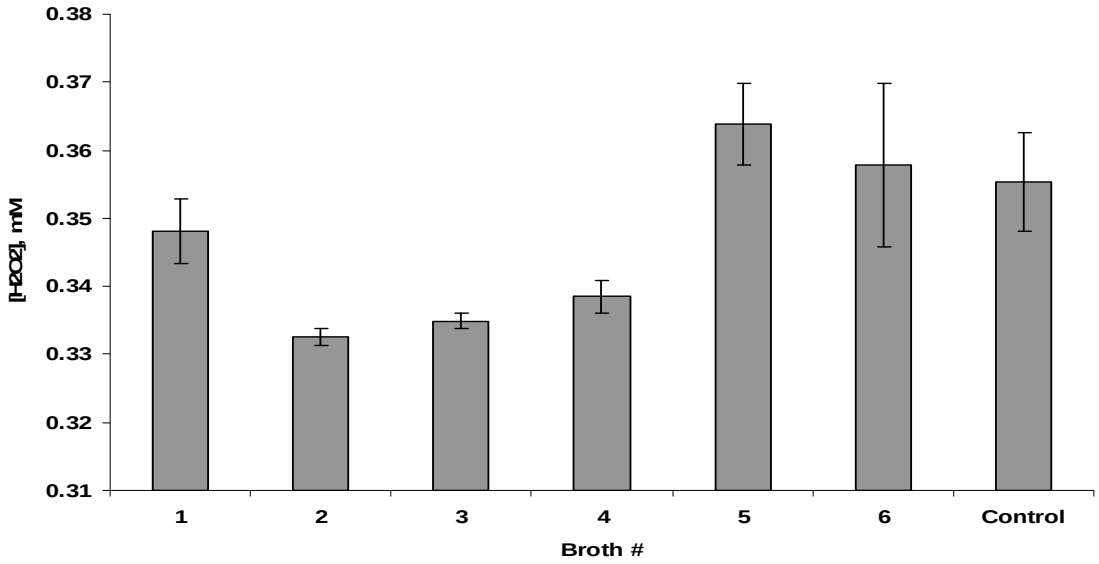
Figure 20. Continual production of H_2O_2 from DP1004 pregrown to stationary phase. Cells were grown microaerobically in CATPGlu in a 125 ml flask, at 37˚C, under ambient light. At OD_{550} = 0.64, the cells were harvested. Concentration of H_2O_2 produced by cells was measured ([H_2O_2] = 2.69 mM) before washing and diluting the cells to various cell densities. The diluted cells were grown separately in a 125 ml flask at 37˚C for 1 hour. Supernatant was harvested from each dilution for H_2O_2 measurement. Bars represent the endogenous H_2O_2 production after dilution with Y-axis the concentration of H_2O_2 produced, while the X-axis represents the dilution factors and the optical density of turbidity after dilution, and after 1 hour incubation.

(f) Determination of the presence of quorum-sensing peptide in the activation of H_2O_2 production.

Induction of pneumococcal competence by competence stimulating peptide (CSP) was found to take place between cell density of 0.05 – 0.15 read at an optical density of 600 nm (7). We therefore checked this property in H_2O_2 production by measuring H_2O_2 concentration present in supernatants of cultures treated with supernatants collected from different growth points at one hour interval from cell density of 0.1 read at an optical density of 550 nm (previous data showed no apparent differences from 600 nm) to stationary phase. A cell density of 0.3 was used for the study because the cells are restored to exponential growth from lag-phase and H_2O_2 production is not activated during this point after inoculation (refer to method and material). No significant effect on H_2O_2 production was observed (Fig. 21A).

We then employed another way to test the possibility of the presence of a quorum-sensing peptide. The H_2O_2 concentration present in supernatants of cultures pretreated with or without proteinase K during aerated growth was determined. Unexpectedly, in both cases, the cells exhibited normal H_2O_2 concentrations regardless of the presence of proteinase K (Fig. 21B). Together, the data confirmed that the connection between quorum-sensing peptide and the H_2O_2 production did not exist.

A.



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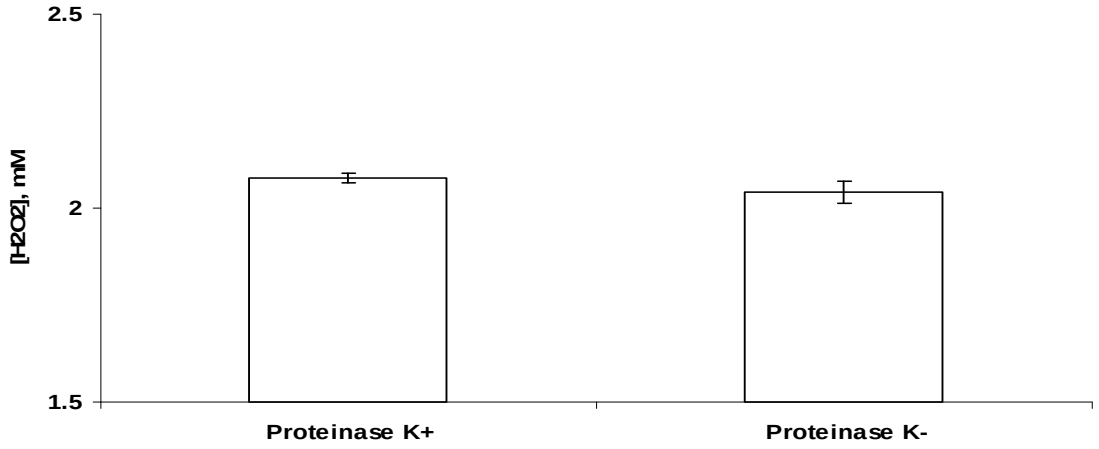

Figure 21. Determination of extra-cellular peptide that induces the ability of DP1004 to produce H_2O_2. (A) Effect of the supernatant, harvested from various growth phases on the ability of DP1004 to produce H_2O_2. Cells were pregrown microaerobically in CATPGlu in a 250 ml flask at 37˚C under ambient light. At OD_{550} = 0.3, 5 ml of cells were aliquot into 125 ml flasks and treated with the supernatant collected previously at various growth phases. Supernatant was harvested after 90 minutes incubation for H_2O_2 measurement. Bars represent H_2O_2 concentration with Y-axis indicates the concentration values while broth # 1 – 6 collected from sample between early growth phase (1) and late growth phase (6) is represented on X-axis. (B) Measurement of H_2O_2 produced by DP1004 treated with proteinase K (K+) or without proteinase K (K-) treatment. Cells pregrown to OD_{550} = 0.32 were aliquot into two 125 ml flasks. Proteinase K was added to one of the flask. The two flasks were incubated aerobically for 1 hour. Turbidity was measured. Supernatant was used to measure the amount of H_2O_2 produced by the cells. Left-bar represents result from proteinase K treated sample while the right-bar represents result from non-proteinase K treated sample. Bars represent H_2O_2 concentration measured from the samples' supernatant with Y-axis indicating H_2O_2 concentration while the type of treatment is represented on the X-axis. Mean values of duplicates from one independent experiment (±SE) are presented. N = 2.

SpxB has been shown to require O_2 for its activity (81). This finding suggested that production of H_2O_2 by the cells might vary when growing in different apparatuses. The H_2O_2 concentration present in supernatants of cultures grown in a test tube or flask was determined. As expected, the H_2O_2 concentrations were found to differ between the two apparatuses by 8-fold higher concentration more in the flask than in the counterpart (Fig. 22). This suggested that the O_2 content in different apparatuses varied and test tube of 13 mm diameter did not allow H_2O_2 production.

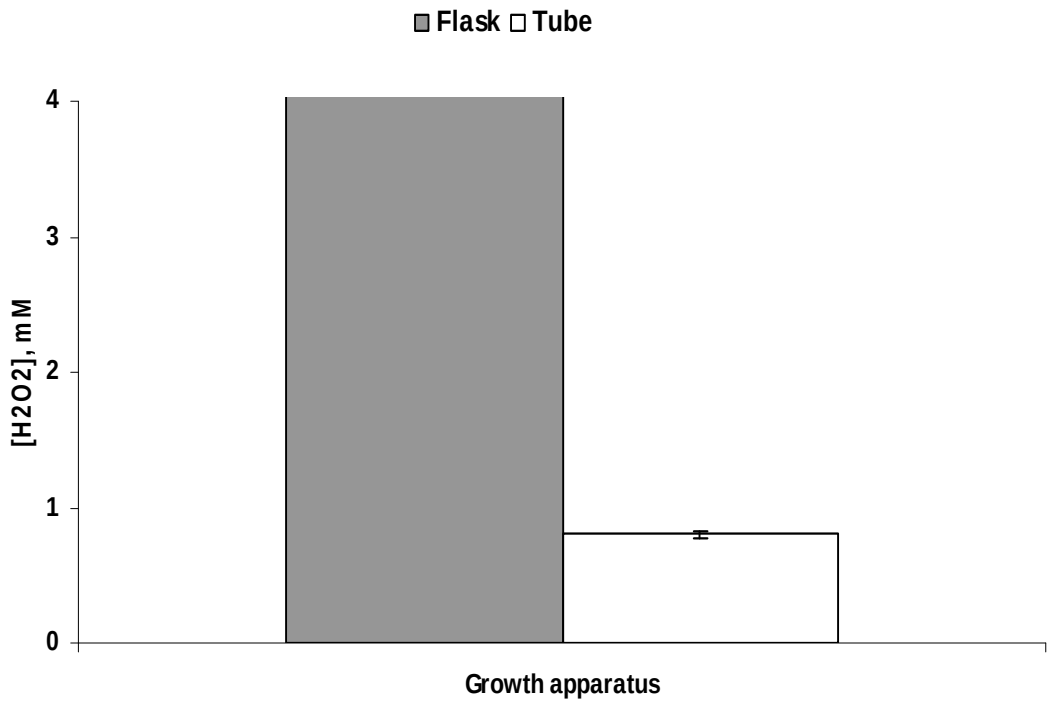

Figure 22. Effect of vigorous shaking on the ability of DP1004 to produce H_2O_2. DP1004 cells were grown microaerobically in CATPGlu at 37˚C, in a 125 ml flask and a 13 mm slip cap test tube. At OD_{550} ~ 0.2, vigorous shaking at 200 r.p.m was supplemented for both tube and flask samples for 1 hour in ambient lighting. Supernatant of each sample was harvested for H_2O_2 measurement. Mean values of duplicates from one independent experiment (±SE) are presented. N = 2.

In a protein cystalization study, SpxB is shown to contain flavin adenine dinucleotide (FAD) (86). It is well-known that light-activated proteins, such as LOV proteins (light-O_2-voltage), are composed of a flavin compound (102). Recently, an association between light and gene regulation has shown to cause virulence in *Brucella abortus* (102). We therefore tested the effect of light on SpxB activity. The H_2O_2 concentration presents in supernatants of cultures grown microaerobically with or without ceiling lights was determined. Indeed, the H_2O_2 production was found to highly present with cultures in the flask under ambient laboratory lighting (Fig. 23). This suggested that light is required for H_2O_2 production.

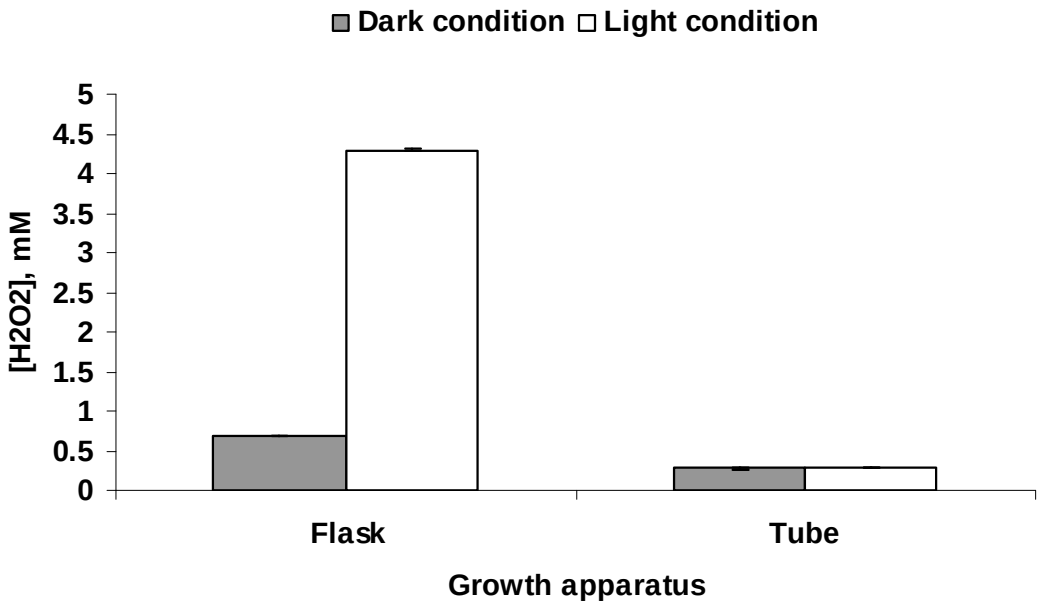

Figure 23. Effect of light on the ability of DP1004 to produce H_2O_2. DP1004 cells were grown microaerobically in CATPGlu in a 13 mm slip cap test tube and a 125 ml flask, at 37˚C overnight. Samples were incubated in the dark incubator or under a light source. Supernatant from each was harvested for H_2O_2 measurement. Mean values of duplicates from one independent experiment (±SE) are presented. N = 2.

We then demonstrated the connection between light and higher concentration of O_2 in H_2O_2 production by determining the H_2O_2 concentration present in supernatants of cultures grown in a test tube of flask after vigorous shaking was supplemented. Surprisingly, the H_2O_2 concentration present in the flask without ceiling lights was restored in the presence of elevated O_2, demonstrating no significant different to the counterpart (Fig. 24). Together, the data suggested that SpxB activity could be governed by O_2 concentration and visible light while higher O_2 concentration supplemented by 200 r.p.m. shaking by-passed the need of light for H_2O_2 production.

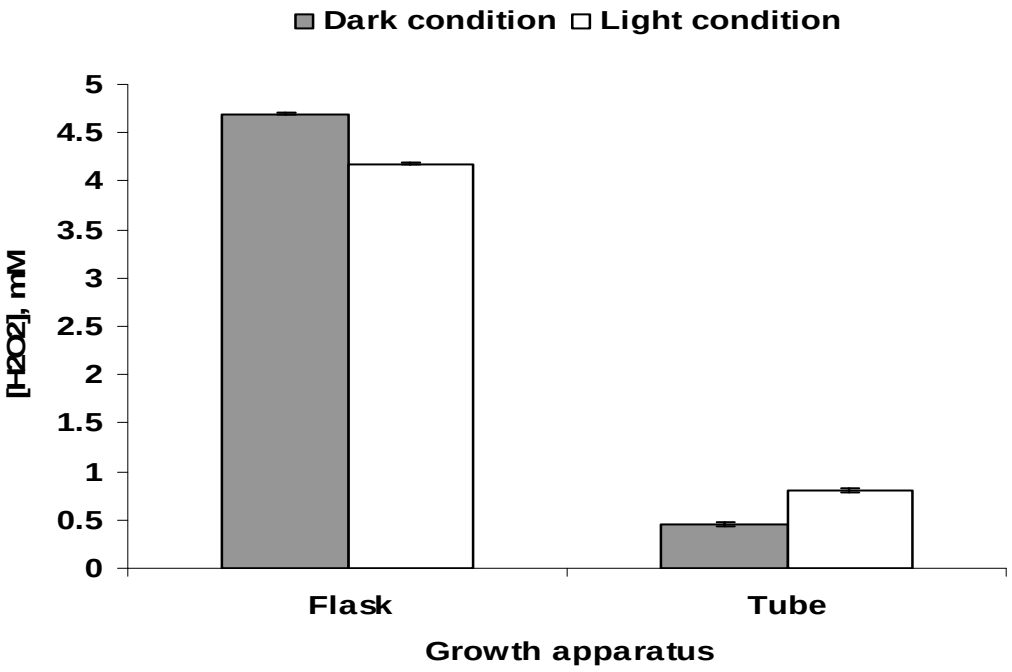

Figure 24. Effect of light on the ability of DP1004, grown under aerated condition to produce H_2O_2. Cells were grown microaerobically in CATPGlu at 37˚C in a 125 ml flask and a 13 mm slip cap test tube. At OD_{550} ~ 0.2, vigorous shaking at 200 r.p.m was supplemented for both flask and tube samples for 1 hour, and either in the dark or under the ambient laboratory lighting. Supernatant from each sample was harvested for H_2O_2 measurement. Mean values of duplicates from one independent experiment (±SE) are presented. N = 2.

CHAPTER V

CONCLUSION

The presence of reactive oxygen compounds in facultative anaerobes is inevitable, especially in the bacterium *S. pneumoniae* that exists primarily in an aerobic environment. Despite that, higher H_2O_2 concentrations found in it, exhibits a novel characteristic of this catalase-negative microbe. In addition, current findings that (a) lactose induced H_2O_2 resistance in *S. pneumoniae* and (b) H_2O_2 resistance has an association with H_2O_2 production, extended the findings by Pericone (81). The aims of the present study involved (a) elucidation of the mechanism of lactose-regulated resistance to H_2O_2 and (b) determination of factors responsible for H_2O_2 production. These were explored by identifying the genes that are up-regulated by lactose as well as involved in H_2O_2 resistance and determining H_2O_2 concentrations by different conditions, respectively. In brief, our experimental findings are demonstrated below:

First part,

(1) The genes spr1639, *ccpA*, *pyrB*, and *truB* are involved in H_2O_2 resistance in *S. pneumoniae.*

Second part,

(2) Mutations of spr1639, *ccpA*, *pyrB*, and *truB* do not have a defect in general cultural characteristics.

(3) H_2O_2 resistance is positively correlated with H_2O_2 production in *S. pneumoniae*.

(4) *spxB* operon is polycistronic.

(5) Negative correlation of *spxB* activity with the expression of the gene was demonstrated simultaneously.

(6) *spxB* activity is negatively correlated with the food availability.

(7) *spxB* activity is positively correlated with the growth phase.

(8) *spxB* activity is non-reversible after committement.

(9) *spxB* activity is positively regulated by light.

(10) *spxB* activity is up-regulated when cells are grown in a flask.

(11) Light-dependence of *spxB* activity can be by-passed by aeration.

Part I. Mechanism of H_2O_2 resistance in *S. pneumoniae*.

Lactose pregrown cells exhibited higher resistance to H_2O_2 than any other sugars tested. This suggested that a lactose-induceable genetic pathway is involved in H_2O_2 resistance. Indeed, there were genes up-regulated by lactose examined by growing cells on X-gal agar. However, only *truB* gene exhibited an increased expression in lactose compared to glucose. For unknown reason, *trmE* was not successfully cloned in *E. coli*. The function of this gene is suggested in *E. coli* to govern cell proliferation (115). The *bgaA* was not further studied here because it has been extensively studied and has been

experimentally shown to be involved in the lactose metabolism of *S. pneumoniae* (117). None of the genes found belong to any of the protein homologues known to be responsible for H_2O_2 resistance. Despite that, mutation of the genes led to decreased resistance to H_2O_2 while complementation restored H_2O_2 resistance in the mutants, only observed in DP1004 background. In fact, complementation of *pyrB*-deficient mutant showed approximately 2-fold higher resistance than the wild-type, further suggesting that *pyrB* was required for H_2O_2 resistance. In addition, this agreed with another finding that destruction of *pyrB* will result in a polar effect to its operon which rendering the cells a reduced oxidative stress (Fig. 27), demonstrating that *S. pneumoniae pyrB* operon (Fig. 26) might have the same function as its close relative, *Lactobacillus plantarum* (Fig. 25, 27) (44, 72). This suggested that there are at least two genes that are involved in H_2O_2 resistance in the presence of lactose, and proposed question is answered.

Mutation of ccp homologues spr1639 and sprr1813 caused H_2O_2 resistance to reduce in the mutants while complementation restored the resistance to H_2O_2 only in spr1813-deficient mutant, suggesting that spr1813 is involved in lactose-regulated H_2O_2 resistance. This is consistent with another finding, demonstrating that spr1813 responds to lactose by up-regulating the *bgaA* of *S. pneumoniae* (56). In addition, regulation of *pyrB* by spr1813 was also suggested. Though no cre site could be detected within the promoter region of *pyrB* operon (44). The reason for failure to restore H_2O_2 resistance in spr1639-deficient mutant, which was previously shown to have at least 4-fold higher expression in lactose than in glucose, was not known. However, it could become another ccp of the bacterium that is involved in H_2O_2 resistance regulated by lactose.

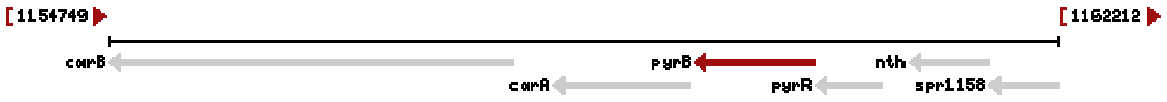

Figure 26. Suggested pneumococcal *pyr* operon, NCBI (71).

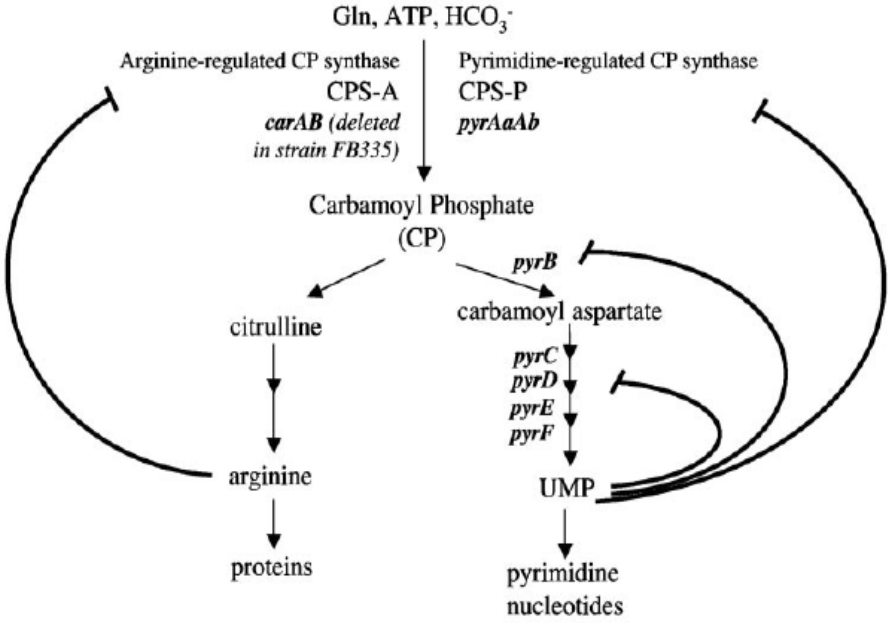

Figure 25. Illustration of pyrimidine and arginine biosynthesis in *Lactobacillus plantarum* (72).

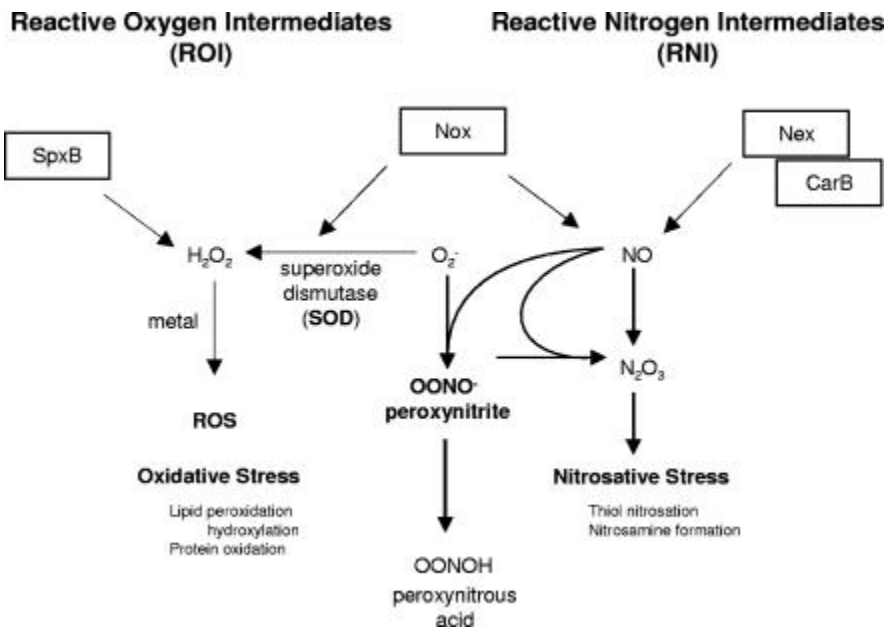

Figure 27. Suggested interaction of H_2O_2 with NO (44).

Overall, our data in the first part of this study do not contradict with current findings, mentioned earlier, except, different results were observed when conditions vary. In addition, our data also added a possible factor of pathogenesis in *S. pneumoniae*. So, further work will be to study the mechanism of pathogenesis using sugar as one of the factors, hopefully, a more complete picture of the mechanism of pneumococcal pathogenesis will emerge.

Part II. Mechanism of H_2O_2 production in *S. pneumoniae*.

Different growth phases exhibited levels of H_2O_2 resistance independent of H_2O_2 production, which was determined by growing cultures in a tube or flask. This finding suggested that growth phases can also govern H_2O_2 resistance. This is consistent with other findings where expression of every gene vary from phase to phase. This is shown in a pneumococcal gene expression profile, demonstrating that more house keeping genes are expressed as growth phases increase (62). An implication is that H_2O_2 resistance during stationary phase is governed by the bacterial detoxification mechanism (62, 98). We excluded the ability of mismatch repair which could have contributed to the resistance by repairing DNA damage from H_2O_2 because the strains we used were *hexA* null mutant (106).

If *S. pneumoniae* has an increased resistance to H_2O_2 during stationary phase, one would expect its *spxB* expression to increase. In contrast, such correlation did not exist as demonstrated previously in two separate strains derived from Rx1. A similar observation

was found in the strain SP1625, which allowed the determination of both *spxB* expression and the enzyme activity, simultaneously. Both suggested that *spxB* expression does not correspond to H_2O_2 production. This agrees with the finding that demonstrates maximal *spxB* expression is present during early growth phase and H_2O_2 concentration is optimum during stationary phase when cells are grown microaerobically (7, 104). This further implied that there is another unknown genetic pathway governed by growth phase that is involved in H_2O_2 resistance.

spr0643 was included in SP1625 construction because previous data demonstrated that it was essential for H_2O_2 production and arranged in the same transcriptional direction to its adjacent gene *spxB*. This suggested *spxB* operon is polycistronic. Co-transcription of both genes examined by RT-PCR agreed with this finding, but, Pericone's finding that *spxB* operon is monocistronic (determined by Northern blot analysis) (81). However, a spr0643-deficient mutant did not have a significant effect on the H_2O_2 production, conferring the gene's function unknown at this point. A protein homolog has been found in *E. coli*, and it is involved in protecting an *E. coli* cell from oxidative stress (32). An H_2O_2 sensitivity test could be performed to determine the similar function demonstrated in *E. coli*. This could be carried out in *S. pneumoniae*, using spr0643-deficient mutant and comparing it with the wild-type to demonstrate the effect of complementation. Preliminary data demonstrated that spr0643 was likely to provide the cells protection against oxidative stress because mutation of the gene demonstrated high sensitivity to 10 mM H_2O_2 exposure (data not shown).

One of the reasons that explains no parallel relationship between *spxB* expression and the enzyme activity is that glucose represses the *spxB* activity, demonstrated as glucose was depleted, H_2O_2 production was activated (104). In addition, the pattern of *spxB* expression and enzyme activity can be explained by the involvement of spxB during competence in early growth phase, where competence is maximally present (7). If the first explaination is true, one would expect H_2O_2 production to reverse in the presence of fresh medium containing glucose because glucose inhibits H_2O_2 production. In contrast, H_2O_2 producing cells washed and grown in fresh medium did not lose H_2O_2 production. Despite that, onset of H_2O_2 production was found at the same growth phase, the stationary phase.

We further explored the possibility that glucose represses H_2O_2 production by determining H_2O_2 concenration present in supernatants in cultures grown in other sugars. Overnight cultures produced normal H_2O_2 concentrations in all sugars except, fructose or galactose, which growth curves showed pneumococcal cells did not grow well in those sugars. This contradicted with Taniai's finding that glucose represses H_2O_2 production (100). However, the data suggests that the glycolytic pathway is necessary for SpxB activity because all cells that showed normal H_2O_2 production undergo this pathway in the sugars tested. This is consistent with SpxB reaction, in that, it requires pyruvate, a product of glycolysis (81). This might have hindered the effect of different sugars on H_2O_2 production in overnight cultures because previous data suggested that increased H_2O_2 production should be found in cells pregrown with lactose. We therefore pursued an experiment to measure H_2O_2 concentration present in supernatants of cultures at various

growth phases. Indeed, cells pregrown with lactose started H_2O_2 production in early growth phase, and the H_2O_2 production was gradually incremented, with maximum H_2O_2 concentration appearing during stationary phase. This was consistent with other finding that H_2O_2 resistance positively corresponds to H_2O_2 production, because previous data demonstrated higher H_2O_2 resistance cells pregrown with lactose (81). An implication is that lactose opens up another pathway of H_2O_2 production, but it is unknown whether that pathway also requires SpxB activity, because lactate oxidase is shown to function as another H_2O_2 producing agent in *S. pneumoniae* (104).

If the other explaination is right, mutation of competence gene would allow early H_2O_2 production, but this is yet to be determined. A preliminary study demonstrated aeration caused reduction of competence and increased production of H_2O_2 (data not shown). Together, the data suggested a connection between O_2, H_2O_2, and competence. Stationary phase-governed H_2O_2 production demonstrad previously in the presence of glucose, suggested the possibility that cell density regulates H_2O_2 production, so that, accumulation of cell density would activate H_2O_2 production. As expected, cell density equivalent to turbidity during stationary phase activated H_2O_2 production. In addition, one would also expect reversed H_2O_2 production from H_2O_2 producing cells when dilution is made. In contrast, dilution of H_2O_2 producing cells from stationary phase did not abolish H_2O_2 production. Together, the data implied that H_2O_2 production is cell-density dependent and not reversible in H_2O_2 producing cells. In addition, another connection could also be established between high cell-density and H_2O_2 resistance, which is yet to be determined experimentally.

A negative connection between quorum-sensing peptide and cell-density dependent H_2O_2 production was demonstrated by growing cells in supernatants from different growth phases followed by H_2O_2 measurement, because quorum-sensing peptide is commonly released from bacterial cells during high cell density (25). A similar finding, performed by aerating cells with or without proteinase K followed by H_2O_2 measurement, demonstrating that the culture did not have a defective in H_2O_2 production, further demonstrated that no quorum-sensing peptide was involved. However, involvement of non-peptide molecules presented on cell surface is possible in H_2O_2 production.

Different apparatuses, tube or flask, used to grow culture was found to lead to different concentrations of H_2O_2. This result suggested that variation in O_2 concentration affects production of H_2O_2 by *spxB*. Indeed, normally, H_2O_2 production is activated simply by providing aeration that supplies an unknown concentration of O_2. In addition, activation of H_2O_2 production in cells grown microaerobically in a flask during stationary phase may also relate to this. Together, the data suggested that certain concentration of H_2O_2 is required for SpxB activity to take place. An implication of that is certain O_2 concentration may contribute to different degrees of pneumococcal pathogenesis, since this pathogen is primarily found in an aerobic environment.

Culture of cells microaerobically grown in a flask under ambient light activated H_2O_2 production. This suggested that light is required for *spxB* activity. This agreed with other finding that flavin-composed proteins are activated by light because SpxB is

composed of flavin (86, 102). A continuous study of light-dependent H_2O_2 production will determine the exact wavelength for the SpxB activation.

Lastly, aerated cells grown in a flask under ambient light exhibited similar concentration of H_2O_2 to the one without light. This suggested that higher O_2 concentration abolishes the light-mediated mechanism (still unknown) that is involved in H_2O_2 production. Together, the data implied that there is an unknown association between light and O_2 by which either both or O_2 is required for H_2O_2 production.

Overall, we showed that H_2O_2 production is not the only factor that offers a protection against H_2O_2 insult. We found that, resistance to H_2O_2 could also be activated as cell-density increases gradually. In addition, role of light in H_2O_2 production may also suggest to us another possible connection between H_2O_2 production and the resistance to H_2O_2 when light is present.

In summary, *S. pneumoniae* acquires ways to protect itself from oxidative stress caused by the environment the bacterium lives in, which is O_2-rich, and to be viable to perform pathogenesis at the degree similar to the condition that is favorable to it. Through the discovery of the connection between O_2, sugar, and light in virulence of *S. pneumoniae*, one can also apply the findings to other lactic acid bacteria to further disclose hindered capabilities of bacteria.

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VITA

Hung King Tiong

Candidate for the Degree of

Master of Science

Thesis: MICROBIOLOGY AND MOLECULAR GENETICS

Major Field: Microbiology and Molecular Genetics

Biographical:

Personal Data:
I was born and brought up in Malaysia, which is a South asian country. The majority of population in Malaysia is Malay, followed by Chinese and Indians. Having been around such diverse/cosmopolitan population I have learnt to converse in Melayu, English which is taught to us since kindergarten and chinese which is my mother tongue. I have completed education till 12^{th} grade in Malaysia.

Education:
Completed the requirements for the Master of Science in Microbiology and Molecular Genetics at Oklahoma State University, Stillwater, Oklahoma in May, 2009.
Completed Bachelor of Science in Plant and Soil Sciences (option: Biotechnology) at Oklahoma State University, Stillwater, Oklahoma in May, 2004.

Experience:
Equipped with molecular techniques helpful for extracting DNA from bacterial or plant cells, performing DNA restrictive recombination, cloning, amplifying DNA by PCR, purifying DNA by gel filtration, performing DNA mutagenesis, performing oral presentation, performing laboratory demonstration, and etc.

Professional Memberships:
American society for microbiology, Graduate student association of microbiology.

Name: Hung King Tiong Date of Degree: May, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: GENETICS OF THE RESISTANCE TO AND PRODUCTION OF H_2O_2 IN *STREPTOCOCCUS PNEUMONIAE*

Pages in Study: 93 Candidate for the Degree of Master of Science

Major Field: Microbiology and Molecular Genetics

Scope and Method of Study:
Streptococcus pneumoniae (Pneumococcus) is a causative agent of severe diseases affecting young as well as older people. Pneumococcus produces very high levels of hydrogen peroxide (H_2O_2) (>1 mM). In the presence of pneumolysin, H_2O_2 has been shown to cause pneumococcal meningitis in humans. H_2O_2 is a by-product of oxidation of pyruvate by the pyruvate oxidase, SpxB, during the generation of acetyl PO_4 in pneumococcus. Acetyl PO_4 then is utilized to make ATP. Current work focuses on manipulating the genome to study the role of H_2O_2 in pneumococcal pathogenesis. Previously, pneumococcal cells pregrown in the presence of lactose were shown to have an increased resistance (10-fold) to H_2O_2 challenge.

Findings and Conclusions:
Random mutagenesis showed that the genes *pyrB*, *truB*, spr1639, and spr1813 are responsible for the resistance. Based on the DNA sequence data, it has been speculated that the *spxB* operon is mono-cistronic and expressed constitutively. Our experimental evidence, however, showed that maximal production of H_2O_2 is linked to the onset of stationary phase under static growth conditions or exposure to molecular oxygen while the transcriptional activity of *spxB* was constitutive even during the exponential phase of growth. Also, insertion of a reporter gene immediately down stream of the *spxB* gene resulted in normal H_2O_2 production implying that glyoxalase, a co-transcript of *spxB*, is not required for H_2O_2 production and other factors besides SpxB are needed for H_2O_2 production in this pathogen. We speculate that the generation of this toxin is linked to either quorum-sensing and/or availability of nutrient resources. Together, the findings helped to expand the pathogenesis pathway of H_2O_2 in *S. pneumoniae*.

ADVISER'S APPROVAL: Dr. Moses Vijayakumar